THE EXPERIMENTAL VASCULAR LESIONS PRODUCED BY BACILLUS MALLEI.

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PLATES VII AND VIII.

In the course of an experimental study of the histological lesions of glanders, the result of which I hope to publish at a later date, I was impressed with the nature and regular occurrence of vascular changes in rabbits and guinea pigs following the injection of *Bacillus mallei* and its poison. The work was not undertaken primarily for the study of the blood vessels, but as it was early observed that the vascular lesions were similar to those produced by the administration of adrenalin and occurring in glanders in man, I considered this part of the histological study sufficiently important to warrant a special communication.

METHODS.

The animals used in this series of experiments were full grown rabbits and guinea pigs. The inoculation was made subcutaneously, intravenously, or intra-peritoneally. The same strain of *Bacillus mallei*[1] was used throughout the work. The original culture was attenuated in virulence by frequent transplantation during a period of eighteen months. Three series of animals were inoculated with definite amounts (1) of an attenuated culture, (2) of a highly virulent culture, and (3) of the cultures killed by heat. The attenuated cultures were obtained as noted above. The highly virulent cultures were obtained by frequent passage of the bacilli through guinea pigs. The killed suspensions were prepared by heating the virulent culture in salt solution to 55° C. The bacilli were prepared for inoculation by growing cultures on slanted

[1] The culture was obtained through the kindness of Dr. Frothingham of Boston.

1 per cent. dextrose-agar for from 24 to 48 hours at a temperature of 37° C.; afterwards washing down the growth and thoroughly emulsifying it in 10 c.c. of normal saline or bouillon. The dosage of the three emulsions was as follows: (*a*) Attenuated culture, 1 c.c. for guinea pigs, 5 c.c. for rabbits. (*b*) Virulent culture, 0.5 c.c. for guinea pigs, 5 c.c. for rabbits. (*c*) Killed culture, 0.5 c.c. for guinea pigs, 1.0 to 1.5 c.c. for rabbits.

Although these were the usual doses employed, another dose of the attenuated bacilli was found useful for the production of a partial immunity in guinea pigs which was to prepare them for larger doses of the virulent culture. These large doses were used to produce certain proliferative vascular changes noted below. The dose in this case was usually 0.5 c.c. of a suspension in which one loopful of a twenty-four hour agar growth had been added to one cubic centimeter of saline. The doses were repeated every third or fourth day over a period of two to four weeks. Rabbits received the injections into the marginal ear vein. Guinea pigs, owing to the smallness of the ear veins, were inoculated into the axillary and femoral veins, aseptic precautions being used. Subcutaneous and intra-peritoneal injections were employed with equally good results. The emulsion of dead bacilli was injected without filtration. The ordinary mallein of the market failed to give results. Occasionally rabbits succumbed to the injection of the living virulent bacilli in from three to five days but this was not the rule; the rabbit is not highly susceptible to the glanders bacillus and easily stands large and repeated doses. Pregnant rabbits and mothers are more susceptible to the inoculations.

Guinea pigs give more constant results than rabbits, although the vascular changes do not differ essentially in the two species. The tolerance to the glanders bacillus was best observed in the female pigs. The difference in susceptibility of rabbits and guinea pigs as regards sex and the bearing of this on glanders immunity has recently been discussed by Nicolle.[2]

For the histological study the tissues were fixed in Zenker's fluid, alcohol or formalin, and embedded in paraffin. Methylene-blue-eosin and phosphotungstic-haemotoxylin were used in the rou-

[2] Nicolle, *Annales de l' Inst. Pasteur*, 1906, xx, 625, 698, 801.

tine staining of sections. Weigert's elastic and Mallory's connective tissue stains were used for the special demonstration of elastic and connective tissues. Sharlach R. was employed tc demonstrate the presence of fat. The methylene-blue-eosin method was more useful as a routine stain than the haemotoxylin-eosin; with the former the early vascular lesions were more readily determined, the calcified areas more distinctly indicated, and the proliferative endothelium and degenerative medial changes were more readily observed. The phosphotungstic-haemotoxylin method was especially useful in marking out the degenerative lesions of the smooth muscle fibres giving to such areas a brownish black color in deep contrast to the faintly stained normal portions of the vessel wall.

The intimal and medial lesions were well differentiated with Mallory's connective tissue stain; indeed this stain alone made possible the separation of the internal elastica of the lesion from the degenerated band of the circular fibres. It stained the internal elastic membrane in the vascular lesion a dark blue in contrast to the normal elastica which was stained a distinct pale blue. The degenerated muscle fibres were stained a deep orange-red in contrast to the purple-blue of the normal muscle cells. The intimal endothelium was stained a salmon color. The medial lesion was stained a deep orange-red owing to some special affinity of the acid fuchsin for degenerating muscle fibres. The deep orange-red of the medial area of degeneration in the immediate proximity to the swollen and straightened dark blue bands of the internal elastica gave a striking picture.

HISTOLOGICAL LESIONS.

For purposes of clearness I will consider the histological lesions under three headings: (1) the exudative, (2) the proliferative and (3) the degenerative. This grouping of the lesions corresponds closely with the degree of the virulence of the cultures employed. In general it can be stated that a highly virulent culture produces vascular lesions of an acute inflammatory type, while a culture of low virulence produces proliferative changes. The degenerative changes are secondary to the proliferative lesion.

While the glanders poison attacks especially the arterial system, and more especially the smaller arteries of the peripheral circulation, none of the arteries wholly escapes injury.

The selection of the smaller arteries by the bacterial poison in preference to the aorta and its larger branches is a striking feature of the two cases of glanders infection studied in man. In both cases the vascular lesions were found in the terminal arteries of 1 mm. to 2 mm. in diameter; and they were especially numerous in the arteries distributed to the skin, lungs, brain and kidneys. The lesion consisted of circumscribed aneurysmal bulgings of the vessel walls showing proliferative intimal and degenerative medial changes (Plate VII, Fig. 1). The diseased arteries were in no way involved in the general infectious process going on in other tissues.

The acute vascular changes occurring in the rabbits and guinea pigs are usually focal in distribution. The most marked lesions occur in the male pig and the female rabbit. Other lesions than the vascular ones, resulting from the injection of *Bacillus mallei,* occur with less frequency.

In animals the earliest change of the vessel noted consists of swelling of the sub-endothelial tissue spaces of the intima due to œdema, the overlying layer of the endothelium remaining perfectly normal. In still other vessels aggregations of polymorphonuclear leucocytes between the intima and the media occur. Often these collections of polynuclear cells are so densely packed as to form layers extending more than half around the circumference. The sub-endothelial collections of leucocytes at the point of greatest density may attain a depth of six to eight cells; while the overlying endothelium shows no break or appreciable protoplasmic or nuclear change. In a longitudinal section of the vessel the sub-endothelial layer of leucocytes may extend, gradually tapering in the long axis of the vessel, for a distance many times that of the circumference. It would appear that the polynuclear zone extends from a primary focus in the direction of the long axis rather than circumferentially. In these vessels showing polynuclear collections, or sub-intimal abscesses, there is a notable absence of fibrin and other cellular elements. The lumen of the vessels involved is often crowded with polymorphonuclear cells, but I was never able to observe them adherent to or penetrating the intimal endothelium. However, one must assume that the sub-intimal lesion results from a wandering out of the leucocytes, and where this occurs in the larger arteries the media offers a barrier to the leucocytic penetration and consequently causes an accumulation of the cells under the intima at some portion of the vessel wall.

Thrombosis is a frequent lesion especially of the vessels at or near the site of inoculation. The ear vessels of the rabbit invariably undergo thrombosis after injection of *B. mallei* or its poison; and not only the vein into which the

material is introduced, but also the other veins and arteries. The thrombosis takes place gradually, spreading from the primary focus which is the point of entrance of the injecting needle. In some animals the greater part of the ear sloughed off. The lesions follow injection of poison or killed bacteria as well as living bacilli. The mesenteric and omental veins are commonly affected after intra-peritoneal injection.

Diffuse or focal infiltration of the media with eosinophile cells appears in the smaller arteries; in one female rabbit dying of acute glanders four days after intravenous injection the media of the mesenteric arteries contains eosinophiles. These collections are focal and apparently invade the vessel wall from the peri-vascular tissue where eosinophiles are present in moderate numbers. Occasionally the eosinophiles are grouped about the vaso-vasorum; in other instances they are collected in areas of well advanced degeneration of the smooth muscle fibers.

Lymphoid and plasma cell infiltration of the vessel wall is rarely met with; it is noted in the omental vessels of two female guinea-pigs after a subcutaneous inoculation.

A peri-capillary lymphoid infiltration is quite common in vessels at or near the site of inoculation.

The proliferative changes are confined exclusively to the intima of the vessels, and are undoubtedly the chief factor in the production of the degenerative changes of the media. The proliferation takes place in the endothelial cell layer and not in the sub-endothelial connective tissue. The degree and extent of intimal reaction depend upon the virulence and dosage of the culture. All grades are seen from a simple heaping up of the cells at one focus, or at many foci, to complete occlusion of the lumen. The proliferated endothelial cells are often phagocytic, containing in their protoplasm red blood cells, altered blood pigment, and fragmented nuclei. This proliferation of the intima is best obtained in the rabbit. In some arteries the phagocytic cells project into the lumen in irregular finger-like masses. Mytotic figures in the attached endothelial cells are often observed. (Plate VIII, Fig. 4.)

In vessels in which a gradual occlusion of the lumen occurs the proliferated endothelial cells undergo degeneration. As the narrowing of the lumen progresses the changes in the occluding endothelial cells advance; finally there is complete necrosis of the cells as indicated by the loss of nuclei and the more or less homogeneous hyaline appearance of the whole occluding mass. In cross sections of small arteries a characteristic appearance is the focal nature of the endothelial proliferation; some parts of the intima show no change while other parts show an enormous "heaping up" of the endothelium. (Plate VIII, Fig. 4.)

The proliferated cells bordering on the lumen are often enlarged to ten times their normal size; direct action of the poison on these border cells may be assumed from the condition of the underlying endothelial cells, which are less than half the size, and are in a good state of preservation. Many of the attached cells bordering on the lumen contain, in addition to large and small fat droplets, numbers of red blood cells, polymorphonuclear leucocytes and nuclear fragments. Frequently these large proliferated cells become detached and are seen in the lumen in a state of marked fatty or hyaline degeneration. These large degenerated cells have been found occluding the sinusoids of the liver and cause

ultimately focal necrosis of the parenchymal cells. Some of the smaller vessels are completely occluded with large detached and degenerated endothelial cells, giving rise to a more or less indefinite hyaline and fatty mass, which mass is not unlike the focal areas of degenerated phagocytic endothelial cells seen in the Malpighian bodies of the spleen in certain acute infectious diseases. (Plate VII, Fig. 3.) This phagocytic action of the intimal endothelium of the vessels occurs with remarkable frequency in rabbits and guinea-pigs following the intravenous injection of a glanders culture of moderate virulence. In addition to the phagocytic property of the intimal endothelium there is a still more striking feature in the occurrence of giant cells, which appear as perfectly formed multinucleated elements having a well defined protoplasmic limitation. (Plate VIII, Fig. 6.) These giant cells may be seen attached along the outermost layer of the endothelial heaps. They contain from four to seven nuclei which are usually situated excentrically. There is no doubt as to the endothelial origin of these elements, a fact of importance in considering the histogenesis of giant cells in general. Still another type of proliferation is that seen in vessels in which the endothelium increases evenly around the whole circumference in the form of concentric lamellæ until there occurs complete obliteration of the lumen. In this form of lesion the endothelium always maintains itself in even layers and at no period, even after occlusion, are the cells found degenerated or in a poor state of preservation as in the case of the obliterating endarteritis resulting from the phagocytic cell occlusion.

In the vessels showing this particular form of obliteration by means of superimposed layers of intimal cells, the musculature and adventitial coats increase proportionately to the intimal increase.

The media attains an enormous thickness, and as there cannot be made out any increase in the number of the nuclei of the media an actual hypertrophy of the smooth muscle fibers probably occurs. The adventitia increases even beyond the thickness of the media. Such vessels now appear as enormously hypertrophied closed tubes, each layer sharply defined and proportionately thickened.

A word may be said about the changes noted in the lymphatics. These vessels are dilated even to many times their normal size. The single lining layer of endothelial cells is swollen and finely granular. In many of these cells the protoplasm is filled with small fat droplets. An occasional large endothelial cell may be found in the lumen together with a large amount of serum. The changes in the lymphatics are especially marked in the omentum and in the abdominal parietes after the subcutaneous and intra-peritoneal injection of *B. Mallei.* Occasionally an endothelial cell lining the lymphatic shows a well-defined mytotic figure.

The degenerative lesions are secondary to the proliferative lesions. They always occur in the innermost layer of the media and in that portion of the vessel wall which shows an intimal heaping up of the endothelial cells. At such points in the media the vessels ranging from 0.5 to 1 mm. in diameter show under the low power of the microscope circumscribed bulgings or sacculations of the wall together with broad bands of degeneration which lie immediately external to the internal elastic membrane. The internal elastic membrane is between the sub-endothelial layer on the inner side and the degenerative lesion

of the media on the outer side. There is absolutely no proliferative or degenerative change in the sub-endothelial layer of the intima. In this situation the internal elastic membrane loses its normal sinuous curves, straightens out and becomes a fusiform band with a thickened central fibrillated portion, and the narrower distal portions many times thicker than the normal membrane. Throughout the lesion it has the appearance of dipping deeply down beneath the intima, but this dipping is only apparent and is due to the fact that the endothelium of the intima is proliferated to form several layers of cells. (Plate VII, Fig. 2.) There is an aneurysmal bulging of the vessel wall at the site of the lesion and this bulging outward of the vessel wall is so great that even the circumscribed heaping of endothelial cells fails to fill the increased convexity of the vessel at this point. The section of one of these smaller arteries resembles in contour the section of an eye in its antero-posterior diameter, the sacculation corresponding to the bulging of the cornea.

The degenerative lesion begins in the circular fibers of the innermost layer of the media at a point directly external to the point of greatest thickening of the intima. Its tendency is to extend along the circular muscle fibers maintaining the same thickness throughout; it is only in the advanced lesions that it spreads to the fibers of the central zone. In the advanced lesions cross sections of the larger vessels often show the degenerated medial band extending beyond the primary lesion. The longitudinal sections also show that the medial degeneration in advanced disease of the vessels may extend beyond the primary intimal lesion.

The earliest change in the smooth muscle fibers occurs in the form of fatty granules grouped about the nuclei. Later these fat particles increase in number and in time coalesce and form large globular masses. The nuclei become indistinct and finally disappear. The fibers undergo rapid degeneration and ultimately calcification. (Plate VIII, Fig. 5.) This medial change may occur at first in a single isolated smooth muscle fiber, or in a small group of fibers immediately external to the elastica interna. This selection of single cells by the glanders poison is comparable to the action of certain toxins which give rise to necrosis of single cells or small groups of cells in the liver.

The degenerative changes in the internal elastic lamina are similar in nature to those occurring in the muscle fibers and occur simultaneously with them.

In no case was there a degenerative process in the media without proliferation of the intima; but proliferation of the intima frequently occurred when there was no change in the media.

DISCUSSION.

The vascular lesions in experimental glanders afford an excellent opportunity to study the early intimal and medial changes in experimental arterio-sclerosis. In addition to the degenerative changes analogous to those produced by adrenalin, the bacillus and its toxin produce lesions of an exudative and proliferative character. The kind and severity of the inflammatory conditions would seem to depend largely upon the virulence of the culture.

Various endarterial lesions, for the most part of a degenerative character, have been described by different workers, but the forms produced by the glanders toxin, especially the inflammatory ones, receive no mention. Some authors have regarded the experimental lesions produced with bacteria as analogous to arterio-sclerosis in man, but this view is not generally accepted, and has been disputed by the investigators of adrenalin arterio-sclerosis.

Gilbert and Lyons[3] were the first to produce experimental aortic lesions by the injection of bacteria and their toxins (1) with, and (2) without mechanical injury to the vessel wall. They speak of the successive giving away of the smooth muscle cells, new formation of connective tissue, and calcification of the elastic fibres. In their experiments the aorta alone showed change, which fact together with the sclero-calcareous nature of the lesions lead them to regard the lesions as analogous to aortic sclerosis in man.

Crocq[4] found it necessary to injure the aortic wall in order to produce arterial lesions by bacterial injections. He employed diphtheria, typhoid, and colon bacilli and streptococci in his experiments. Pernice[5] was unable to obtain with the diphtheria bacillus and the staphylococcus lesions in the aorta without first injuring the wall by mechanical means. He especially emphasized the occurrence of a " round cell " infiltration about the vaso-vasorum. Thérèse[6] experimented with bacterial products and describes " round cell " infiltrations of the peri-vascular tissue and capillaries which he attributed to the effect of toxins.

Although changes in the intima and media have been produced separately and described by numerous experimenters with bacteria, no mention appears in the literature, so far as I can find, of medial changes following and dependent upon primary intimal lesions, which occur with such frequency in the vessels of experimental glanders. Workers with bacteria in the experimental production of arterio-sclerosis describe lesions occurring in the aorta but do not mention peripheral vascular changes. It is now well

[3] Gilbert and Lyons, *Archiv de med. exper.*, 1904, xvi, 13; *Compt. rend. Soc. de Biol.*, 1889, xli, 583.

[4] Crocq, *Arch. de med. exper.*, 1894, vi, 583.

[5] Pernice, *Atti del R. acad. delle Scienze med. in Palermo*, 1895.

[6] Therese, Thesis, Paris, 1893.

recognized that in man sclerosis of the peripheral vessels is not infrequently found when there is no demonstrable lesion in the aorta. Moenkeberg[7] described calcareous lesions spreading distally in the vessels of the human extremities while the aorta and its main branches remained unaffected.

The injection of cultures of *Bacillus mallei* of low virulence almost invariably gives rise to lesions in the smaller arteries, while the aorta and its larger branches remain free even from microscopic changes. On the other hand a highly toxic killed culture produces aortic as well as peripheral lesions. The repeated administration of the autolyzed product of highly virulent glanders bacilli produces marked proliferative and degenerative changes in the walls of the aorta and the smaller vessels. These facts would seem to indicate that the aorta and its larger branches are better able to resist this bacterial poison than the peripheral arteries.

Erb[8] states that the adrenalin lesions often occur in the arteries supplying the various organs, and draws attention to those in the renal arteries.

The vascular lesions in the two cases of fatal glanders infection in man were of the sub-acute type, the patients having died of a glanders septicaemia one after six and the other after eight weeks of illness. In these two cases of human glanders, the disease manifested itself as a bronchopneumonia followed by a papular and pustular skin eruption.

In them the aorta and its larger branches were remarkably free from visible sclerosis, showing only a few scattered yellow streaks along the arch and in the abdominal aorta just before its bifurcation. The remarkable features of both cases are the focal vascular lesions of the smallest arteries, especially those of the lungs, muscles and skin. These vascular lesions are by no means confined to the areas of inflammation but are found throughout the peripheral arterial system.

Microscopically the earliest lesions show a focal heaping of one portion of the intimal endothelium. Immediately external to this intimal proliferation the internal elastic membrane becomes straight-

[7] Moenkeberg, *Virchow's Archiv*, 1903, xi, 141.
[8] Erb, *Archiv fur exper. Path.*, 1905, liii, 173.

ened and somewhat swollen. Associated with the intimal heaping there is often a narrow band of degeneration of the innermost layer of the media which lies in close proximity to the intima and separated from it only by the swollen internal elastic membrane.

In the experimental animals, as well as in the human cases, the microscopic study of the peripheral vessels reveal focal changes in the smaller arteries of the internal organs. In general the lesions consists of a circumscribed area of proliferation or "heaping up" of the intima with a corresponding well defined band of degeneration in the smooth muscle fibres of the media. This area of degeneration is always situated in both human and experimental disease in the "inner portion" of the media.

Almost without exception the workers with adrenalin describe the medial changes in the aorta of rabbits as situated in the "central zone" of the middle coat (Josue, Fischer, Pearce and Stanton, Lissauer, Klotz).[9] They explain the difference in situation of the changes in man and in rabbits by the difference of the aorta in man and in rabbits. The intima of the human aorta is a much more complicated structure than the intima of the rabbit's aorta. It contains the so-called musculo-elastic layer. The differences, however, between the smaller arteries of man and of the rabbit and guinea pig are so small that they may be disregarded. The glanders poison produces identical lesions in the peripheral vessels of man, the rabbit and the guinea pig.

Great stress has been laid upon the extensive proliferation of the intima in human arterio-sclerosis which is said to be absent in experimental adrenalin lesions in rabbits. The vascular changes produced by the injection of adrenalin into rabbits have been entirely of a degenerative character and have been described as occurring in the "middle zone" of the media. Adrenalin it seems rarely if ever gives rise to any proliferative change in the intima. The glanders lesion, on the other hand, is caused by primary proliferative changes in the intima of the peripheral vessels, followed by secondary degenerative changes in the media.

[9] Fischer, *Deutsche med. Woch.*, 1905, xxxi, 1713. Pearce and Stanton, *Jour. Exper. Med.*, 1905, viii, 74. Lissauer, *Berlin. klin. Woch.*, 1905, xlii, 675. Klotz, *Jour. Exper. Med.*, 1905, vii, 633; 1906, viii, 504; 1906, viii, 322.

Of the many workers who have approached the question of arterio-sclerosis with experiments on animals, one group has confined their efforts to the injection of bacteria and their toxins with or without first injuring the inner wall of the vessel; another group has injected substances such as nicotine (Adler and Hensel)[10] and adrenalin. The results obtained by both groups of observers have attracted much attention but they have been so varied as to leave us still in doubt as to the origin of arterio-sclerosis. A number of workers have produced proliferative intimal lesions in the aorta by the injection of certain bacteria and their toxins, or lesions of a purely degenerative nature in the media; but nowhere can I find both these types of lesions described as the result of the action of any one species of bacterium and its toxin.

In the vascular lesions produced experimentally by the injection of the glanders bacillus and its toxin we have intimal lesions associated with changes in the media analogous to similar combined changes produced by this bacillus or its poison in the blood vessels of man. Not only are the intimal and medial changes associated but they occur in such a manner as to leave no doubt that the intimal lesion is primary and that the medial lesion is secondary. The degeneration of the media is the result of the proliferative intimal change. This first evidence of degeneration in the experimental lesions and in the human lesions occurs in the "innermost zone" of the media.

CONCLUSIONS.

1. *Bacillus mallei* and its poison produce a variety of vascular lesions in the rabbit and the guinea pig.

2. The type of the lesion depends upon, (*a*) the virulence of the culture, (*b*) the sex of the animal and (*c*) the degree of acquired immunity.

3. The vascular changes of a proliferative and degenerative nature produced by the slow action of the glanders poison in rabbits and guinea pigs are analogous to the vascular lesions caused by sub-acute glanders infection in man.

4. The most common site of the glanders vascular lesions of animals and man is the peripheral vessels, and especially the smaller visceral arteries.

[10] Adler and Hensel, *Jour. Med. Research*, 1906, xv, 229.

5. The aorta is a less common site of the experimental lesions.

6. The vascular lesions produced experimentally by *Bacillus mallei* and its poison consist of three processes, (*a*) exudation, (*b*) proliferation, (*c*) degeneration.

7. The lesions produced by sub-acute glanders in man consist of two processes, proliferation and degeneration.

8. The primary reaction of the vessels in experimental animals and in sub-acute human glanders consists of a proliferation of the endothelium of the intima.

9. The first degenerative changes observed in experimental animals and in sub-acute human glanders occur in the "innermost layer" of the media and not in the so-called "middle zone."

10. The cause of the degenerative change in the inner layer of the media appears to be interference with the nourishment of the circular muscle fibres of the media by proliferation of the endothelium of the intima.

FIG. 1.

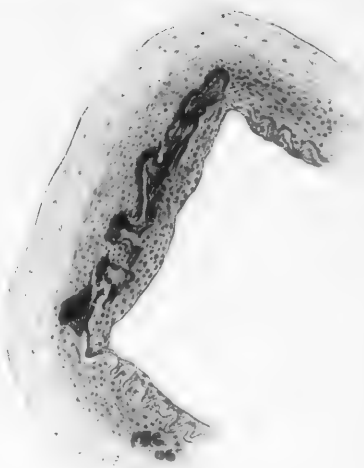

FIG. 2.

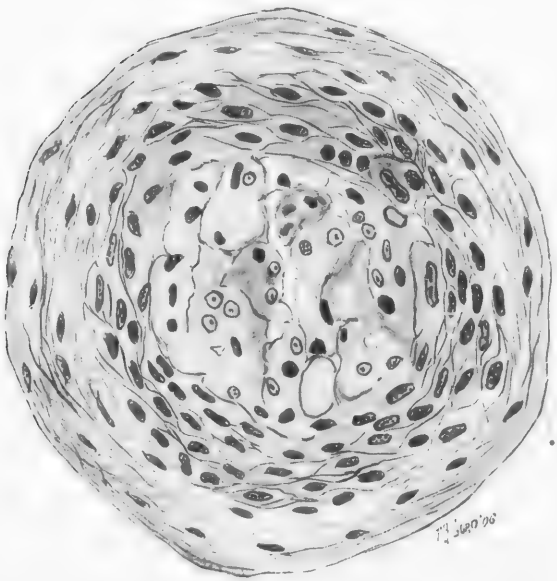

FIG. 3.

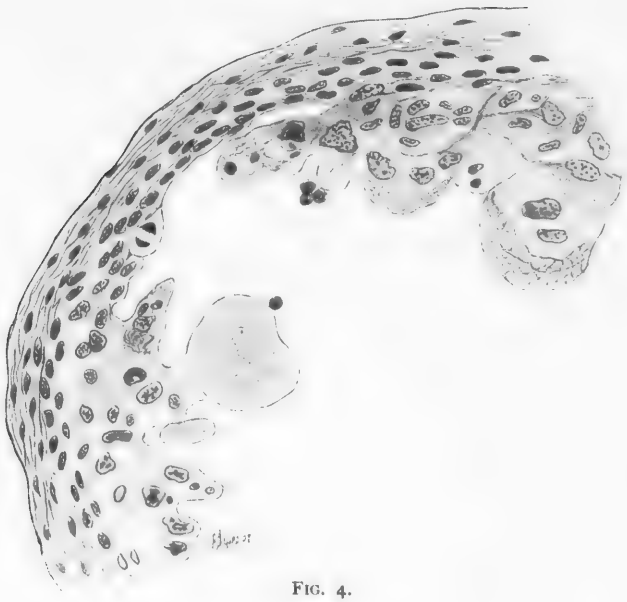

FIG. 4.

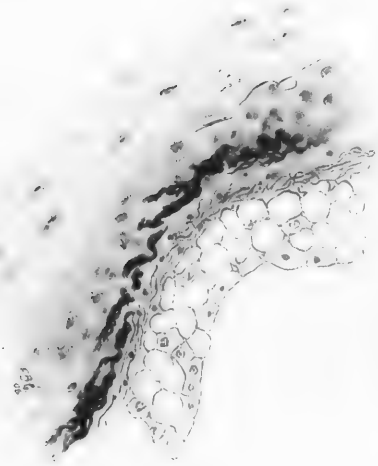

FIG. 5.

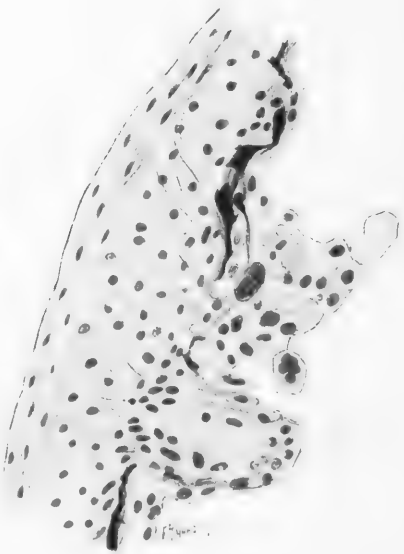

FIG. 6.

Explanation of Plates VII and VIII.

Fig. 1. Cross-section of human artery 2 mm. in diameter, from a case of fatal glanders infection, stained after Mallory's connective tissue method. There is to be noted a definite circumscribed bulging of the vessel wall, in which the lesion consists (1) in the "heaping up" of the intima, (2) the straightened and swollen elastica, and (3) the "innermost" layer of medial fibers in a marked state of degeneration. Degenerated fibers are stained a deep orange-red and are in close proximity to the diseased internal elastica.

Fig. 2. Cross-section of a rabbit's artery 2 mm. in diameter stained with methylene-blue-eosin, showing lesion following the repeated intravenous injection of *B. mallei.* Note the zigzag calcified degeneration of the internal elastica and the innermost fibers of the media. The lesion is immediately external to and associated with the intimal proliferation.

Fig. 3. Cross-section of a small artery of a rabbit stained with methylene-blue-eosin, which shows almost complete obliteration of the lumen with the proliferated intimal endothelium. Many of the innermost cells are seen as detached phagocytes undergoing fatty and other degeneration changes. The innermost proliferated cells of the endothelium are compressed into layers and show good preservation.

Fig. 4. Section of a small artery of the rabbit stained with methylene-blue-eosin. Note the enormous size of the proliferated endothelial cells, which are phagocytic and undergoing fatty degeneration. Also note the attached endothelial cell undergoing mitosis.

Fig. 5. Small artery of the guinea-pig showing (1) marked vacuolation of the "heaped up" intimal endothelium, (2) calcified degeneration of groups of smooth muscle fibers of the innermost portion of the media.

Fig. 6. Small artery of the guinea-pig stained with methylene-blue-eosin. Section shows focal proliferation of the intimal endothelium with the formation of "giant cells." Also shows degeneration and calcification of the internal elastica and innermost fibers of the media.

ELECTRIC CHARGES CARRIED BY THE HEMOLYSINS.

By OSCAR TEAGUE and B. H. BUXTON.

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Field and Teague[1] have recently shown that toxins and antitoxins under the influence of the electric current migrate toward the cathode, and in a second series of experiments they found that the agglutinins of typhoid immune serum also travel toward the cathode.

With hemolysins the conditions are somewhat more complicated than with toxins or agglutinins owing to the presence of two factors, amboceptor and complement, being necessary to complete the hemolytic system. It has been shown by Lillie[2] and Henri[3] that red corpuscles migrate toward the anode in an electric current. Provided, therefore, that neutralization of electric charges is a factor in the combinations necessary to effect hemolysis, one would suppose that the immune bodies should travel towards the cathode and the complement toward the anode. We have indeed determined that hemolytic immune bodies migrate in the direction of the cathode, but our experiments indicate very strongly that complement also travels in the same direction.

The immune serums were procured by inoculation into rabbits, of ox and horse corpuscles, washed free of serum, and suspended in an equivalent amount of salt solution, the blood being drawn from the ear or the carotid after intraperitoneal injection of thirty to forty cubic centimeters of the corpuscles in individual doses of five to six cubic centimeters. The immune serums are designated as rabbit v. horse and rabbit v. ox. For complement fresh normal rabbit serum has been used throughout. The accompanying tables refer, some to rabbit v. horse, and some to rabbit v. ox serum, but

[1] *Jour. of Exp. Med.*, 1907, ix, 86.
[2] *Am. Jour. Phys.*, 1903, viii, 273.
[3] *Compt. rend. Soc. de Biol.*, 1904, lvi, 867.

the results with both kinds of serum have been practically identical. There have been slight variations from one experiment to another, but variations of degree only, not of kind, and the tables have been selected from the more typical experiments.

The first cell used was similar to that devised by Field and Teague for their experiments with toxins and antitoxins, and is

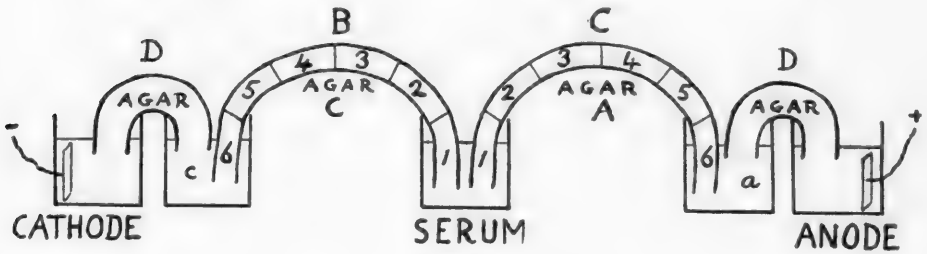

FIG. 1.

shown in Fig. 1. But instead of the platinum electrodes used by them we substituted copper and zinc, so that by surrounding the copper electrode (cathode) with copper sulphate and weighing it before and after the experiment we were able to determine the average current passing through the cells. By surrounding the zinc electrode with zinc sulphate, polarization was avoided, and a more uniform current obtained. The ordinary lighting circuit of 110 volts was used throughout. In Fig. 1 the semicircular tubes *A, C, D, D,* contain one per cent. agar made up with physiological salt solution, and salt solution is also used in Cells *a* and *c,* while the center cell contains the serum to be tested. In order to be certain that no change in the reaction of the central parts of the apparatus should occur during the passage of the current the two outer agar tubes, *D, D,* were made slightly alkaline and a trace of phenolphthalein added. It was found that a considerable amount of acid travelled from the anode and quickly decolorized the anode tube, *D,* which was therefore changed several times during the course of each experiment. Repeated tests showed that under these conditions, there was no perceptible change in the reaction of the three central cells, or in the agar tubes *A* and *C*. Our first

plan was to suspend the red corpuscles in the agar of the tubes *A* and *C* and watch their progressive destruction as the current carried the antibodies from the serum through the agar in one direction or the other.

Test.—Sensitized red corpuscles (ox or horse) suspended were in the agar *A, C,* and fresh normal serum (complement) in center cell of Fig. 1. The result was negative. No destruction of red corpuscles occurred in either of the agar tubes. Repeated and varied experiments on these lines always ended in failure. The red corpuscles in the agar tubes never showed any signs of hemolysis.

The next plan adopted was to fill the agar tubes *A* and *C* with plain agar made up with salt solution, and, after passing the current for four or five hours with serum in the center cell, to cut up the agar into pieces indicated by the numbers in Fig. 1. Each piece of agar was then rubbed down in a mortar, extracted over night in the ice chest with four cubic centimeters of salt solution, and the extracts tested next day for their hemolytic properties. This procedure was the same as that adopted by Field and Teague for their experiments with toxins.

In making the tests given in the accompanying tables we have always used a total of 1.5 cubic centimeters of fluid in each test tube composed of:

1. The immune serum,	.5 c.c.
2. The complement serum,	.5 c.c.
3. Five per cent. suspension of the corpuscles.	.5 c.c.

If therefore we find in the tables that complement serum 0.3 dilution has been used, the final dilution in the test tube is 0.1, or one third of the dilution as given in the table. The same applies to the immune serum, the final dilution being always one third of that given in the tables.

Experiments with cell shown in Fig. 1.—Agar extracts.

Table I.—In center cell is inactivated (56° C.) immune serum. Agar extracts are tested next day with complement. The cathode extracts, 1, 2 and 3, hemolyze the red corpuscles, indicating that the immune bodies travelled into the semicircular agar tube, *C,* in the direction of the cathode. The anode extracts, on the other hand, are not hemolytic.

Table II.—In center cell is fresh immune serum, *i. e.,* complement and immune bodies together; the agar extracts are tested next day with red cells. The results are negative. The hemolytic principles of the fresh immune serum do not appear to travel together through the agar.

Table III.—The extracts of Table II are treated with complement. The table shows that the cathode extracts, 1 and 2, contain immune bodies which have travelled towards the cathode, but the complement of the fresh immune serum has not travelled with them, or Table II would have shown positive results at the cathode end.

Table IV.—In center cell is fresh normal serum. Agar extracts are tested next day with inactivated immune serum. The results are negative. There is no indication that the complement of the fresh normal serum has travelled in either direction.

Conclusions with Cell I.—Immune bodies travel toward the cathode, but there is no indication that complement travels in either direction.

Since the results with complement had so far been negative, certain possible reasons for failure were taken into consideration.

1. During the experiments the agar tubes became warmed owing to resistance to the electric current. The heating might be sufficient to destroy or weaken the complement.

2. Complement may be absorbed by the agar.

To meet the first of these possible reasons for failure the cell was modified in such a way that the serum and the agar tubes could be packed in ice during the course of the experiment. Fig. 2 shows this form of cell, the principle being the same as with Cell 1, but with the serum in a closed tube attached by rubber joints, *J J*, to the semicircular agar tubes.

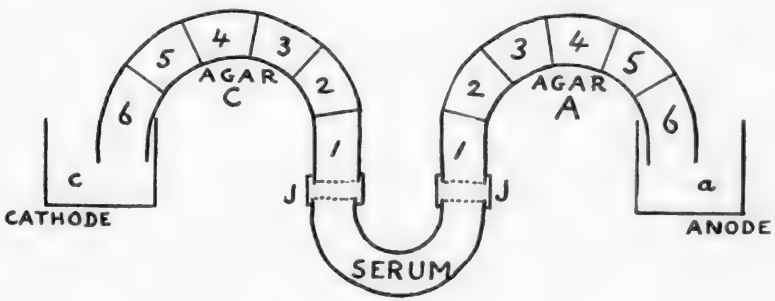

FIG. 2.

With this cell immune bodies could again be shown to migrate readily towards the cathode, but, as with the first cell, tests with complement were always negative. No complement could ever be demonstrated in the agar extracts. It has seemed probable, therefore, that the complement is absorbed by agar.

It has for some time been well established that complement is absorbed and neutralized by organ cells, bacteria, yeast, specific precipitates, and similar substances even though the complement may have no apparent action upon the agents of its absorption. Among the more recent experiments are those of Landsteiner and Stankovich,[4] who demonstrated absorption of complement by various inert substances in suspension or in colloidal solution, kaolin, casein, glycogen, and certain lipoids, such as cholesterin. Table V shows that agar also has this neutralizing action on complement. Fresh normal serum, after treatment with agar has no complementary action on immune serum, whereas the same serum merely filtered through gauze is quite active.

To eliminate the absorption of complement by agar several other cells were devised and tested, but the only one which afforded satisfactory rsults (Fig. 3) consists of a glass tube divided into three compartments by two stopcocks. The whole of the tube is filled with the serum to be tested, and the two outer chambers are connected by a rubber joint, *J*, with curved agar filled tubes; the distal ends of the curved tubes being dipped into cells containing salt solution and corresponding to Cells *a* and *c* in Fig. 1. While attaching the curved agar tube to the cell it is necessary to stick a small capillary glass tube into the rubber to allow some of the serum to escape. If this precaution is not taken the increased pressure will force the agar out of the curved tube. Once the

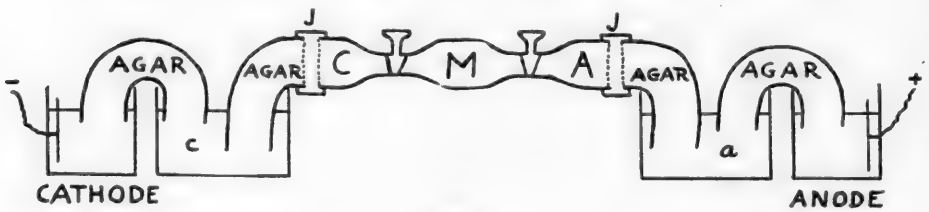

FIG. 3.

agar tube is in position the capillary can be taken out and the minute hole in the rubber closes up of itself. During the passage of the current the cell is packed in ice to prevent heating.

[4] Cent. für Bakt., 1906, xlii, 353.

After the current has passed for four or five hours the stopcocks are closed, and the contents of each chamber *A*, *C*, *M*, collected separately and tested for complement content.

Table VI shows that on addition of immune serum the fluid from the cathode chamber, *C*, is distinctly more hemolytic than the fluid from the anode chamber, *A*, while the fluid from the central chamber *M*, holds an intermediate position. It appears from the table that the *C* fluid is always just one dilution ahead of the *A* fluid, so we may conclude that it contains approximately twice as much complement. It is probable, therefore, that complement has a tendency to migrate towards the cathode, and may consequently be regarded as carrying a positive charge.

There is, however, the possibility that the amboceptors of normal serum form a loose combination with complement, so that the complement might be dragged along in the current by the amboceptors, itself remaining passive. But the balance of evidence is decidedly against the probability of any such combination, and, without discussing the subject in detail we may simply say we do believe that it occurs with either hemolytic or bacteriolytic amboceptors.

In the course of a number of experiments with this cell the chamber, *C*, has always contained approximately twice as much complement as the anode chamber, *A*, although occasionally the center cell, *M*, shows as much as, or even slightly more complement than *C*. We are inclined to attribute this occasional slight excess in *M* to some absorption of the complement in *C* by the agar with which it is in contact.

As a control for the experiments with complement serum we have also made some tests with immune serum in Cell III, as shown in Table VII. We find from this table that, on addition of complement, the fluid in chamber *C* is approximately twice as hemolytic as that in chamber *A*. This corresponds very closely with the relative strength of the fluids observed in the experiments with complement of normal serum.

Since we already know that immune bodies travel towards the cathode, and under the conditions afforded by Cell III, do not migrate more readily than complement, we may regard the experi-

ment as going far towards confirming the view that complement also tends to travel in the direction of the cathode.

Hemagglutinins.—Field and Teague[5] have shown that typhoid agglutinins migrate towards the cathode, and in the course of our experiments with hemolysins we have been able to demonstrate the same for hemagglutinins, as shown in Table VIII. The cathode extracts agglutinate red corpuscles but the anode extracts do not.

Summarizing all experiments so far as they have gone, we may observe that Field and Teague have shown that toxins and antitoxins (diphtheria and tetanus) tetanolysin, antitetanolysin, and typhoid agglutinin travel towards the cathode. The experiments detailed here show that hemolytic amboceptors, hemagglutinins, and probably also complement travel towards the cathode. Apparently, then, all the active principles of serum concerned in antireactions have a tendency to migrate towards the cathode.

TABLE I.

Serum rabbit v. ox (56° C.) undiluted. Hemolytic value 0.001.
Current for five hours. Amperes, 0.036.

Extracts.—Agar tubes divided into six pieces. Each piece extracted with four cubic centimeters of salt solution, kept on ice overnight, and tested next day with complement.

Agar Extract 0.5 c.c.	Complement.	Ox Cells.	Hemolysis in	
			3 Hours.	24 Hours.
Anode extract 1.	0.3	0.5 c.c.	—	—
" " 2.	0.3	0.5	—	—
" " 3.	0.3	0.5	—	—
" " 4.	0.3	0.5	—	—
Cathode extract 1.	0.3	0.5	+++	+++
" " 2.	0.3	0.5	+++	+++
" " 3.	0.3	0.5	+	++
" " 4.	0.3	0.5	—	—
Salt solution.	0.3	0.5	—	—

TABLE II.

Fresh immune serum rabbit v. ox undiluted. Hemolytic value .001.
Current for five hours. Amperes, 0.034.
In each tube: Agar extract, 0.5 c.c.; salt solution, 0.5 c.c.; ox cells, 0.5 c.c.

Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.	Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.
Anode extract 1.	—	Cathode extract 1.	—
" " 2.	—	" " 2.	—
" " 3.	—	" " 3.	—
" " 4.	—	" " 4.	—

[5] *Jour. of Exper. Med.*, 1907, ix, 222.

TABLE III.

Serum and current as in Table II.

In each tube: Agar extract, 0.5 c.c.; complement serum at $\frac{1}{10}$ dilution, 0.5 c.c.; ox cells, 0.5 c.c.

Agar Extract 0.5 c.c.	Hemolysis in		Agar Extract 0.5 c.c.	Hemolysis in	
	1 Hour.	24 Hours.		1 Hour.	24 Hours.
Anode extract 1.	—	—	Cathode extract 1.	+++	+++
" " 2.	—	—	" " 2.	+++	+++
" " 3.	—	—	" " 3.	—	—
" " 4.	—	—	" " 4.	—	—

TABLE IV.

Fresh normal serum, undiluted.

Current four hours. Amperes 0.035.

In each tube: Agar extract, 0.5 c.c.; immune serum rabbit v. ox at $\frac{1}{10}$ dilution, 0.5 c.c.; ox cells, 0.5 c.c.

Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.	Agar Extract 0.5 c.c.	Hemolysis in 24 Hours.
Anode extract 1.	—	Cathode extract 1.	—
" " 2.	—	" " 2.	—
" " 3.	—	" " 3.	—
" " 4.	—	" " 4.	—

TABLE V.

Normal serum $\frac{1}{10}$ dilution, mixed with pieces of crushed agar, and allowed to stand overnight in ice chest. Next morning filtered through gauze.

In each tube: Filtrate, 0.5 c.c.; rabbit v. horse serum ($\frac{1}{10}$ dilution), 0.5 c.c.; horse cells, 0.5 c.c.

	Dilution.	Hemolysis in			Dilution.	Hemolysis in	
		1 Hour.	3 Hours.			1 Hour.	3 Hours.
Serum 0.2 dilution filtered through gauze.	1/1	++	+++	Serum 0.2 dilution + agar and filtered through gauze.	1/1	—	--
Ditto.	1/2	Trace.	+	Ditto.	1/2	—	—
Ditto.	1/4	—	—	Ditto.	1/4	—	—

Fresh normal serum after treatment with agar has no complementary action on immune serum, whereas the same serum merely filtered through gauze is active.

TABLE VI.

Stopcock cell, Fig. III, containing normal serum, $\frac{1}{10}$ dilution
Current for four hours. Amperes, 0.02.

	Fluid from Compartments.	Dilution of Fluid.	Immune Serum R. v. Horse.	Horse Cells.	30 min.	1 Hour.	4 Hours.
1	Anode A.	1/1	.1	0.5	+	+++	+++
2	"	1/2	.1	0.5	—	+	++
3	"	1/4	.1	0.5	—	—	Trace.
4	"	1/8	.1	0.5	—	—	—
5	Cathode C.	1/1	.1	0.5	+++	+++	+++
6	"	1/2	.1	0.5	+	++	+++
7	"	1/4	.1	0.5	—	+	++
8	"	1/8	.1	0.5	—	—	Trace.
9	Center M.	1/1	.1	0.5	++	+++	+++
10	"	1/2	.1	0.5	—	++	+++
11	"	1/4	.1	0.5	—	—	+
12	"	1/8	.1	0.5	—	—	—
13	Salt sol.		.1	0.5	—	—	—
14	Salt sol.		Salt sol.	0.5	—	—	—

TABLE VII.

Stopcock cell, Fig. III, containing immune serum, rabbit v. ox, dilution $\frac{5}{1000}$.
Current four and one half hours. Amperes, .02.

	Fluid from Chambers 0 5 c.c.	Dilution of Fluid.	Normal Serum 0.5 c.c.	Ox Cells.	Hemolysis in		
					1 Hour, 30 Min.	2 Hours.	3 Hours.
1	Anode A	1/1	0.075	0.5	+++	+++	+++
2	"	1/2	0.075	0.5	+++	+++	+++
3	"	1/4	0.075	0.5	++	++	+++
4	"	1/8	0.075	0.5	Trace	++	+++
5	"	1/16	0.075	0.5	—	—	—
6	"	1/32	0.075	0.5	—	—	—
7	Cathode C	1/1	0.075	0.5	+++	+++	+++
8	"	1/2	0.075	0.5	+++	+++	+++
9	"	1/4	0.075	0.5	+++	+++	+++
10	"	1/8	0.075	0.5	+	++	+++
11	"	1/16	0.075	0.5	—	Trace	++
12	"	1/32	0.075	0.5	—	—	—

NOTE.—The fluid of the center chamber was not appreciably different in hemolytic power from that of the cathode chamber.

TABLE VIII.

Hemagglutinin.—Cell, Fig. II, with rabbit v. horse serum (56°), undiluted. No complement added.
Current four hours. Amperes, 0.27.

	Agar Extract 0.5 c.c.	Horse Cells	Agglutination in 30 Min.	Agglutination in 3 Hours.	Agar Extract 0.5 c.c.	Horse Cells.	Agglutination in 30 Min.	Agglutination in 3Hours.
1	Anode extract 1	0.5	—	—	Cathode extract 1	0.5	++	+++
2	" " 2	0.5	—	—	" " 2	0.5	+	+++
3	" " 3	0.5	—	—	" " 3	0.5	—	+
4	" " 4	0.5	—	—	" " 4	0.5	—	—

RESULTS OBTAINED BY THE INJECTION OF PLACENTA INTO ANIMALS OF THE SAME AND OF DIFFERENT SPECIES.

By ROBERT T. FRANK, A.M., M.D.,

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Our anatomical knowledge of the various stages of pregnancy, except in its very earliest period, appears to be nearly complete. The clinical data dealing with the physiology and pathology of pregnancy and the puerperium are also numerous and well understood; but when we seek to obtain information about the agencies which govern the organism during the many changes incident to gestation, we meet with nothing but conjecture and hypotheses. The numerous, but often disconnected facts, upon which these various hypotheses have been founded, are based upon observations obtained at the bed side, the autopsy table, from chemical researches and animal experimentation. In view of these widely differing sources, it is not surprising that contradictions and gaps are numerous.

The object of this study has been to investigate experimentally whether actual proof can be found, that the chorionepithelium (the placenta) exerts a specific influence, whether trophic or other, upon the maternal organism. Two ways of demonstrating such an influence are at our disposal. The one consists of the injection of placental tissue into females of the same species, followed by examination of their generative organs, in order to ascertain whether any anatomical changes, particularly such as are found during gestation, have been produced. The other method is the injection of placental tissue into animals of the same and of other species with the object of producing a specific biological reaction, which can be demonstrated by various methods. In order to acquaint the reader with the current ideas entertained on this subject it will be necessary to refer to some of the literature.

It is generally conceded that the ovary instigates and regulates the periodical menstrual cycle, which is physiologically interrupted only during pregnancy and lactation until the woman reaches her menopause. According to the Born-Fränkel theory (1) which, however, from recent investigations must be accepted with some reserve, the corpus luteum exerts a powerful influence upon the nidation of the ovum. Halban (2) thinks it likely that the ovary and the corpus luteum elaborate products which are antagonistic to one another. When in the right proportion (this would be during gestation), each directly neutralizes the actions of the other. During this time, therefore, some other influence would have to bring about the numerous changes observed. Halban ascribes to the placenta this temporary governing or trophic function. Many observations have definitely shown that nervous influences play no rôle (section of the cord, lactation in transplanted breast tissue, etc.,).[1] Halban has accumulated numerous clinical observations upon which he has built up an ingenious working theory. The placenta, according to this author, causes the hypertrophy and hyperemia of the uterus and of the neighboring organs. In response to the same stimulus the decidual reaction occurs in the uterine mucosa and sometimes in other tissues, and likewise occur the hyperemia and hypertrophy of the breasts. When the placental influence is removed, as after labor or abortion, the pelvic organs undergo a marked involution, whereas on the other hand the previously hypertrophied but inactive breasts begin to secrete. The socalled "menstruation" of the newborn, and the "milk" in the breasts of infants are also ascribed, by the same author, to the withdrawal of the placental influence. Many facts and interesting observations are adduced in this paper, but on the whole Halban's theory has not met with general acceptance. The sole experimental confirmation is that of Miss Lane-Claypon and Starling (3) who

[1] Eckhard, "Beiträge zur Anatomie und Physiologie," *Eckhard's Beitrage,* 1858, i, 1, sectioned the nerves leading to the breast in goats, and found that the secretion of the milk went on unchanged.

Ribbert, *Archiv für Entwicklungsmechanik,* 1898, vii, 688, transplanted the breasts of a young guinea-pig into subcutaneous pockets in the ears. During a subsequent pregnancy the transplanted organs were found to contain functionating acini.

found that the extract derived from a rabbit's placenta, or fetus, repeatedly injected into virgin rabbits produced an hypertrophy of the breasts.[2] The incomplete experiments I have performed along somewhat similar lines will be referred to later.

Another group of investigators have sought for the true explanation by means of anatomical investigation. Chief among them is Schmorl (4) who has with great regularity found trophoblastic elements in the maternal organs. Rarely in normal women, almost regularly in eclamptics, he has discovered emboli, composed of syncytium, in the blood vessels of the lung. Kollman (5) has shown that syncytium undergoes dissolution when subjected to the maternal tissue fluids.

A third group of investigations are based purely upon experimental work, seeking to apply the Ehrlich side-chain theory to solve the problem. The activity in this line of research is so great that new observations are constantly cropping up, and consequently older conceptions will require frequent revision or rejection. Some of the older work has been made doubtful by more recently acquired knowledge. Much of this work unfortunately is still quoted and used in support of theories and far reaching conclusions.

Basing their ideas on the observations of Schmorl and Kollmann, Scholten and Veit (6) set out to prove experimentally, that fetal syncytium is dissolved by the maternal organism. It had been known that the organism reacts to the injection of foreign cells by elaborating a cytolysin which brings about the disintegration of the invader. Animals (rabbits) were injected with human placenta; their serum dissolved placental cells in vitro. Liepmann, who will be referred to immediately, was unable to obtain similar results.

Kawasoye (7) found that human placental cells when placed in the serum of gravid women produced a slight cloudy precipitate and underwent a partial solution. His controls which were kept in sodium chloride solution were, however, quite inadequate.[3]

[2] Lane-Claypon and Starling believe that the placenta and the fetus elaborate an *hormone* which produces the breast changes. They passed the fetal or placental maceration through a Berckfeld filter and injected the fluid.

[3] Kawasoye's experiments may be divided into three groups:

1. A placental antiserum was obtained by injecting human placenta into rabbits. Large placental injections caused death, small ones albuminuria; a

When it first became known that the injection of foreign (artfremdes) proteid produced a reaction in the living organism and could be demonstrated by a visible precipitate, many investigations along these lines were undertaken. If proteid (serum, organs, red blood cells) of a certain species are repeatedly injected into an animal of another species, the serum of the immunized animal becomes cloudy when the serum or the organ extract of the animal used to produce the immunization is added in the proper proportion. The value of this reaction is generally accepted and it has great forensic importance [Uhlenhuth (8)]. It was not only thought, at first, that the reaction applied to the proteid of a given species, but it was believed that a given organ produced an even more specific serum. This refinement has, however, not stood the test of further research, and reaction only for species can in most instances be looked for [Rostoski (9), Sata (10)]. Liepmann (11) announced that rabbits injected with human placenta produced a serum, which contained precipitins for placenta or placental blood. If either of these tissues were added to the placental immune serum, a precipitate was formed. In a later communication (11) he acknowledged that the washed placenta used for immunization necessarily contained general body proteids (connective tissue, red blood cells) as well as the special placental cells, and that, to exclude a general "human reaction" a previous partial precipitation with the serum of a non-gravid subject and clearing by centrifugalizing was required, to exclude this source of error. Several other investigators [Weichardt (12), Opitz (13), Wormser (14)] have obtained negative results in repeating Liepmann's work; while Kawasoye and R. Freund (15) agree with his findings.

Veit (16) in an interesting monograph has based an elaborate theory which seeks to account for all the phenomena of pregnancy,

tolerance could be obtained. Precipitine reaction was positive with the blood of gravida, with retroplacental and cord blood, and with placental extract but negative with blood from the non-gravid and males.

2. The blood of gravid women dissolved placenta in vitro.

3. Albuminous urine of gravid women also produced an antiserum when injected into rabbits. The action of this serum was weaker and less specific than that of placental antiserum.

both those in the mother as well as those of the fetus, largely upon the above quoted works of Scholten, Kawasoye, Liepmann, Kollmann, and Schmorl. The main conclusions, germane to this branch of the subject, are of such general interest that they cannot be omitted.

In broad outline Veit's explanation is as follows: Schmorl has demonstrated syncytium in the lungs of the mother. Liepmann has furnished the biological proof that syncytial constituents are present in the blood of pregnant women, and this fact substantiates Veit's assumption that the placental proteid follows the laws of antitoxin formation and conforms to the side chain theory. Kollman has shown anatomically that syncytium is dissolved by the maternal blood; this solution presupposes a solvent chemical substance in the blood—a lysin. Veit bases all that is to follow upon the assumption "*that there is a continued entrance of syncytium from the intervillous space into the veins of the mother, and a subsequent lysis of the syncytium*" from the very beginning of pregnancy to its termination. Veit's own investigations (17) have convinced him that purely physico-chemical processes (differences in osmotic pressure) do not account for the absorption of salts and proteids by the fetus. Absorption is attended to by the villi, which dip into the maternal blood and show a selective affinity for certain substances only. This selective activity has been demonstrated by Ascoli (18) who proved that egg albumen though present in the blood of the mother did not appear in the circulation of the fetus, and by Schenk (19) who noted quantitative differences in the hemolysins and agglutinins of mother and child.

A possible explanation, according to Veit, is that the nucleus of the syncytium may control the function of assimilation, and the cell protoplasm that of excretion. When the syncytial cell is filled with excretory substances it is cast off from the villus and falls a prey to solution in the maternal blood.

The absorption of iron, which is not in solution, but is an integral part of the hemoglobin molecule and is enclosed by the red blood cell, is brought about by a similar reaction. The lysin acts as an intermediary between the red blood cell and the syncytium, and when this action occurs in the intervillous space the erythro-

cyte is bound to the functionating syncytium of the villus. Thus hemoglobin is removed without producing general hemolysis. Hemoglobin has actually been demonstrated in the villi by Hofbauer (20).

Referring to the pathological phenomena of pregnancy Veit offers the following explanation: The albumin found in the urine of patients suffering from albuminuria of pregnancy, is a true heterologous albumen, such as Liepmann pronounced it to be; it is due to dissolved syncytium which has entered the maternal blood stream in large amounts. If the quantity is very great, the syncytium which is then only partly neutralized acts as a toxin on the kidneys, and produces an actual nephritis. The proof of the nature of this albumin lies in the fact that the specific precipitine reaction is obtained with such urines (Kawasoye). When the syncytium is in greatly excessive amount it no longer acts as a merely local poison, a renal irritant, but produces systemic symptoms which manifest themselves in the eclamptic seizure. Possibly other morbid conditions of pregnancy, such as hyperemesis, icterus, morbus maculatus, etc., are due to this same cause.

The views of Veit, as set forth in the article which has been mentioned, cover every branch of the subject, and in building up his hypothesis he has made use of practically all the work, which has been done along these lines. In the first place he has accepted the anatomical findings of Schmorl and Kollmann.[4] The discoveries of Schmorl may be considered conclusive.

The biological experiments, which are believed to show that the syncytium not only can, but does enter the maternal circulation, acts as a toxine on the tissues of the mother, and stimulates receptor formation, have not been convincing. In opposition to the positive findings of Veit and Scholten, Liepmann, and Kawasoye, Weichardt, Opitz and Wormser obtained negative results, as already stated. Veit has apparently not attached sufficient weight

[4] The work of Kollmann, as far as it bears upon this point, is not convincing. The material was obtained from a fetus three weeks old; some specimens were from pregnant monkeys. He interprets the vacuolization and disintegration noted in the syncytium, as due to lytic processes. It may be said, that changes, such as he pictures, are to be found in almost any rapidly multiplying tissue, and certainly do not necessarily imply any specific solvent process.

to the negative findings, particularly if we take into consideration the fact that some of the positive results were not constant or strictly specific.

Without entering into the merits of Veit's interesting hypothesis, which may yet prove to contain the true explanation of the phenomena of pregnancy, I have sought to repeat some of the work performed, and have used in part the methods already employed by others, in part newer or different methods.

1. To investigate Halban's placental theory three rabbits were repeatedly injected with fresh placenta of pregnant rabbits removed by operation just before term. These animals were used: (*a*) anatomically to study the uterus, ovary and tubes; (*b*) biologically to test the serum for precipitines. The difficulty in obtaining sufficient material for injection made it necessary to cut short this series of experiments somewhat summarily.

2. To control the work performed by Liepmann, etc., three groups of rabbits, each group composed of two animals, a male and a female, were injected repeatedly with the following materials: (*a*) A solution of nucleo-proteid obtained from the human placenta. This was tested for precipitines, and for deflection of complement by the method of Neisser and Sachs. (*b*) A maceration of human placenta made blood-free by washing in plain running water; it was tested by the same methods. (*c*) A maceration of human placenta made as bloodless as possible by washing with large quantities of normal saline solution; it was tested for precipitines, deflection, cytolysis, etc.

RABBITS INJECTED WITH PLACENTA OF RABBITS.[5]

A double purpose was kept in view in this series of experiments. Firstly, if placental tissue should prove to have an inherent specific action such as Halban ascribes to it, repeated intraperitoneal injections ought to produce distinct changes in the genital system. Such changes in the mammary glands were reported by Lane-Claypon and Starling a number of months after my experiments were

[5] This group of experiments was performed in the Pathological Laboratory of the College of Physicians and Surgeons during the months of April and May, 1906. I desire to express my obligations to Professor T. M. Prudden for the courtesies extended to me.

completed. The duration of pregnancy in the rabbit covers thirty days, therefore it would seem necessary to extend the injections over a period of about that length.

Secondly the use of the serum obtained, if potent, would avoid a disturbing factor not to be overcome if the injections were made into an animal of another species, namely hemolysins, and "a general species reaction," for the tissues of the animal used to produce immunization. The work of Kraus and Ludwig (21), and still more recently that of Schultz (22), shows that iso-hemolysins cannot be obtained in rabbits. It might be supposed that experimentally, at least, iso-syncytiolysins, would also not be formed. However the analogy is not quite exact, for in virgin animals (such as were used in this series) the placenta, if it differ biologically from other tissues of the body is a foreign substance. An article by Ed. Martin (23) has just appeared reporting attempts to prove the presence of iso-hemolysins and iso-agglutinins obtained in the following way. Twelve rabbits were operated upon, one half of the pregnant uterus (which in the rabbit is didelphous) being removed, and 0.5 grams of the mashed placenta at once injected into the ear vein of the same animal. In none of the experiments were either hemolysins or agglutinins obtained.

Technic.—Three young female rabbits were employed. A number of other rabbits were allowed to be impregnated at regular intervals, and the gravid uterus taken out at about the twenty-second to twenty-fifth day of gestation. The six to eight placentæ obtained at each operation were at once cut up and mashed in a mortar, the maceration thinned out with an equal volume of normal salt solution and ten cubic centimeters injected intraperitoneally through a large needle into the experimental animals. Throughout, aseptic precautions were employed and no infection occurred. After each injection the animals did not take food for a few hours, but showed no other ill effects; however, during the entire course of the experiments they lost some weight. Nine injections were given to each rabbit at three-day intervals. On the fifth day after the last injection the animals were bled to death and the clear serum obtained in the usual way. The pelvic organs were at once placed in five per cent. formalin and prepared for examination by embedding in celloidin.

The material for histological examination consisted of blocks taken from at least four levels of the tubes and uterus, and longitudinal sections of each ovary. The controls consisted of material taken from normal animals killed in the laboratory during the time in which these experiments were performed. The result of

the examination of these organs can be summed up by stating that there was not the slightest difference between the organs of the injected and of the control animals. No decidual reaction, not even an increased vascularity, was found. Macroscopically no difference in size or appearance could be noted.

The serum tests were performed as follows:

Fresh serum from the injected rabbits was taken in increasing dilution (see Table), placed in small test tubes, and to this serum was added extract of rabbit's placenta (allowed to stand forty-eight hours and prepared in the cold) of various strengths. In another series a loop full of maceration of washed rabbit's placenta was added to the immune serum. Both series were at once brought to a uniform volume of two cubic centimeters by adding normal salt solution. They were placed in the thermostat for one hour and then in the ice-chest for from twelve to twenty-four hours. In none of these series did a precipitate appear.

Anti-serum.	Placental Extract or Placental Tissue		
0.2 c.c.	0.1 c.c.	0.05 c.c.	0.01 c.c.
0.1	"	"	"
0.05	"	"	"
0.2	—	—	—
—	"	"	"

RABBITS INJECTED WITH HUMAN PLACENTA.[6]

Placental Nucleo-Proteid.—The work of Bierry and Meyer (24) and that of Beebe (25) and others lead one to expect that the nucleo-proteid, of a tissue, yields a more specific immune serum and gives less "general species reaction" than the serum obtained from injecting the washed organ. Levene (26) however says, that extracts made from various constituents of red blood cells and used for immunization, either give no hemolytic sera or sera far less potent than those obtained from the entire red blood cells. Pearce and Jackson (27) in a paper published recently, claim that a repetition of Beebe's work has given negative results. These investigators have made a very complete series of experiments from

[6] Almost all the human material used for these and the succeeding experiments was obtained through the kindness of Dr. George Ryder, resident obstetrician of the Sloane Maternity Hospital.

which they conclude that the nucleo-proteids act as mild toxic agents, affecting chiefly the excretory organs, and that they do not produce specific anti-sera. They say that Ehrlich and Morgenroth have shown that anti-sera are produced not by specific cells, but by specific free receptors. Specificity in the morphological sense cannot be demonstrated. The sole criticism that can be applied to the work of Pearce and Jackson is that they have prepared the nucleo-proteid by the "hot" method, which necessitates bringing the mashed organ to the boiling point! Such a method is inadvisable if biological reactions are to be employed. These authors did not attempt to make precipitine tests, but studied the effect of the injections upon the organs in vivo and also histologically.

Technic.—Many (ten to twelve) placentæ were passed through a meat machine and washed in large quantities of 0.9 per cent. saline solution. The blood-free tissue, to which a 0.5 per cent. sodium carbonate solution in the proportion of 1:3 had been added, was placed in the ice chest for twelve hours. Microscopical examination of the last wash water still showed the presence of red blood cells in small number, about one hundred being present in the ordinary low power field. The extract was next filtered through several thicknesses of gauze and the nucleo-proteid precipitated by means of a slight excess of acetic acid. The precipitate was now washed by decantation and attempts made to redissolve it with sodium carbonate solution. It was found that unless the process was hastened, the carbonate solution did not act as a solvent, but a decinormal sodium hydrate caused solution. Reprecipitation and redissolving completed the process.

No exact analysis of the amount by weight represented by each unit of solution was determined, but as from twenty-five to thirty cubic centimeters of very concentrated solution were given, at each injection, a considerable quantity of the nucleo-proteid was used.[1] Dr. P. Levene of the Rockefeller Institute had the kindness to make a Phosphorus determination on the nucleo-proteid used in these experiments. The phosphorus percentage proved to be 0.35.

The solution was injected into two rabbits, a male and a female, intraperitoneally at intervals of about five days, seven injections in all being given to each animal. Serum was taken after the fifth injection and again after two additional injections. The animals flourished during the course of the experiments and gained in weight.

The serum of these rabbits was tested, while fresh, with placental extract (human placenta) and with bits of washed placenta, just as in the previous series. No precipitin reaction was obtained, nor did human blood-serum produce any result.

[1] The first set of sera obtained from these animals were inactivated at 55° C. Noguchi's paper (*Journal of Experimental Medicine*, 1906, viii, 726) refers to the substances which he calls "protectines," or antilysines, formed when sera are heated above 56° C. To avoid all possibility of error from this source the subsequently obtained sera were all inactivated at 52° C.

Another portion of each serum was inactivated at 55° C., for fifteen minutes and then tested by the method of Neisser and Sachs (28) for deflection of complement. This method is regarded as even more delicate than the precipitin reaction, although it is more troublesome and is somewhat capricious.

The rationale of the reaction is as follows: An indicator consisting of an independent hemolytic system is used. In my experiments rabbit's serum made lytic to hen's corpuscles by repeated injections of the washed corpuscles of hen's blood was employed. This anti-hen's serum was inactivated by heat. The other component of this series was hen's corpuscles diluted to a strength of five per cent. If fresh guinea-pig's serum (complement) is added, hemolysis is complete within one hour at a temperature of 37° C.

The second or main components of the reaction are the following: The placenta anti-serum (inactivated) is supposed to contain the amboceptor or specific binding-body if such is elaborated. The organ extract used for immunization must contain corresponding receptors; it is called the antigen. If to these two bodies combined in the proper proportion (see Table) a sufficient but not too great quantity of complement is added, the amboceptor should serve to bind the complement to the receptors. As the amboceptor is specific, it will serve only to bind complement to corresponding receptors. In other words, should an appropriate antiserum be obtained, the free complement is bound to the receptors, and when the hemolytic series (which contains no complement) is added, no hemolysis results. As actually performed, the amboceptor, antigen and complement are added in varying proportion, placed in the thermostat for one hour to allow deflection of complement to take place, and then the second series (hen's antiserum and hen's corpuscles) are added. The complete mixture is then again placed in the thermostat for two hours more. The result, *i. e.*, hemolysis complete, incomplete or absent, is read. The tubes are put in the ice chest for from twelve to twenty-four hours and the final reading taken.

In my experiments various quantities of the components were tried. The following table shows the proportion which was most economical of anti-serum and yet gave the greatest number of proportions of amboceptor and antigen. Throughout, just sufficient complement was used to assure complete hemolysis. The necessary quantity of complement was determined anew each time fresh complement was used.

To these and to all succeeding reactions enough normal salt solution was added to bring the total volume to four cubic centimeters.

When negative results were obtained in reactions similar to those described in the above tables, other experiments were performed, using as much as 1.0 c.c. of anti-serum, and 0.5 c.c. of placental extract. Only after repeated trials of these various proportions, was a negative result accepted.

Anti-serum (Amboceptor).	Placental Extract (Antigen).	G. P. Serum (Complement).	Hen's Anti-serum.	Hen's Corpuscles 5%.
0.2 c.c.	0.01 c.c.	0.04–0.025 c.c.	0.01 c.c.	1.0 c.c.
0.1	"	"	"	"
0.05	"	"	"	"
0.01	"	"	"	"
0.005	"	"	"	"
0.001	"	"	"	"
0.0005	"	"	"	"
0.2	—	"	"	"
0.2	—	"	—	"
—	0.01	"	0.01	"
—	—	"	"	"
—	—	"	—	"
—	—	—	0.01	"
—	—	—	"	"

Placental Extract.	Anti-serum.	G. P. Serum.	Hen's Anti-serum.	Hen's Corpuscles.
0.1 c.c.	0.1 c.c.	0.04–0.025 c.c.	0.01 c.c.	1.0 c.c.
0.05	"	"	"	"
0.01	"	"	"	"
0.005	"	"	"	"
0.001	"	"	"	"
0.0005	"	"	"	"
—	"	"	"	"
—	"	"	—	"
0.1	"	"	0.01	"
0.1	—	—	0.01	"
—	—	0.04–0.025	"	"
—	—	—	"	"
—	—	—	"	"

Before proceeding to the complete reactions, which the tables show, it was necessary to determine in each case that the given anti-serum was inactivated and was neither in itself hemolytic or anti-hemolytic. Two of the anti-sera (those obtained from human placenta washed with normal salt solution) proved to contain a small trace of *amboceptor for hen's corpuscles.* This disturbing factor was removed by treating these sera, for one hour at 37° C., or over night at 0° C., with a great excess of hen's corpuscles and using the supernatant clear fluid after centrifuging. Variously prepared extracts were tried. One set was obtained by placing the macerated washed placenta, diluted with normal salt solution 1 : 3 for forty-eight hours in a shaker, the other in the thermostat for forty-eight hours. A second set of extracts were made using plain distilled water. As no great differences were found, the extract finally used was the one prepared with salt solution in the thermostat. Toluol was added to prevent bacterial growth, and

this antiseptic was later removed by evaporation. The extract was always tested for its hemolytic action and a quantity far below this point used in the reactions.

The four nucleo-proteid sera obtained from the two injected animals showed no deflection of complement. In other terms, the presence of a specific antibody could not be demonstrated either by the precipitin or by the reaction for deflection of complement.

Placenta Washed in Running Water.—As not all the red blood cells could be removed by washing the placenta on a filter with normal salt solution, and it was not feasible to obtain the placentae so early that they could be exsanguinated by washing through the umbilical cord, the finely chopped placenta was placed in a large gauze bag and attached to the cold water faucet. By these means the tissue became practically blood free in a short time, the last wash water showing very few red blood cells, or rather "shadows." The drawbacks attached to this method were fully realized, but it was hoped to control the results by experiments which follow.[8] The placental tissue was then ground in a mortar with sand, and a thick, yet finely divided placental suspension prepared with sterile salt solution. Cultures of each batch of suspensions either showed no growth, or in the case in which growth occurred, an apparently non-pathogenic saprophyte was found.

Three rabbits, two males and one female, were used. One male died after the second injection, autopsy showing a peritonitis due to accidental perforation of the intestine. The other two animals received, respectively, six and eight injections intraperitoneally. The one which received eight injections was given 15 c.c. each time; the other was given from 25 to 30 c.c. The first animal gained over 200 grams in weight, the second lost nearly 400 grams.

The two sera obtained were tested both by the precipitin reaction and by the method of deflection of complement. *The results were absolutely negative.*

Placenta Washed in Normal Salt Solution.—The previous ex-

[8] Washing the tissue with a solution which was not isotonic made it not unlikely that at least some of the antigen would be carried away. Yet by this method a more complete removal of the red blood cells was obtained, and the microscopical examination of the tissue showed that enormous quantities of well preserved placental cells remained for injection.

periments, in which the placenta was rendered practically blood free, at the cost of losing at least some of its active constituents, resulted negatively. As a check and control, the placentae in the series now to be detailed, were first chopped fine with a meat machine, and then each placenta was washed on a filter with ten liters of salt solution, with constant stirring. A careful lookout was kept for all visible blood clots and these were removed. The last wash water appeared clear macroscopically, but under the microscope, from twenty-five to one hundred red blood cells to the low power field were noted. I have been unable to find any reference, in the literature, to the minimum quantity of blood necessary to produce an immune serum. The further treatment of the material in no way differed from that employed in the previous series.

Technic.—Three rabbits were used at the outset, two males and one female; but one male was so seriously injured in a fight with the others that the injections had to be discontinued.

Both the remaining rabbits received nine injections each, at intervals of about five days, from twenty-five to thirty cubic centimeters being introduced intraperitoneally. The animals were bled after the seventh and again after the ninth injection. Both gained markedly in weight during the treatment. In spite of the injections, the *female conceived and bore four normal young* during the course of the experiment. When bled to death she was found to be again pregnant (about twelve days gravid).

Of the four sera, the one serum obtained from the female (after the seventh injection) was rendered useless by being overheated, due to a faulty thermometer. The three other sera were tested for:

1. Precipitin reactions.
   - *a.* Precipitin reactions with placental extract.
   - *b.* Precipitin reactions with cord blood serum.
   - *c.* Precipitin reactions with retro-placental blood serum.
   - *d.* Precipitin reactions with normal blood serum from a male.
   - *e.* Precipitin reactions with placental extract after saturation with male blood serum and with human red corpuscles.
   - *f.* Precipitin reactions with urine (non-albuminous) of gravidæ.
   - *g.* Precipitin reactions with urine (albuminous) of gravidæ.
   - *h.* Precipitin reactions with urine of a male, containing albumin.
2. Reactions for deflection of complement.
   - *a.* Reaction for deflection of complement with placental extract.
   - *b.* Reaction for deflection of complement with normal human blood serum (male).
3. Agglutination reaction with human corpuscles.
4. Hemolysis reaction with human corpuscles.
5. Cytolytic reactions.

a. Cytolytic reactions with emulsions of placental cells.
b. Cytolytic reactions with small pieces of placental tissue.

The results obtained by the various tests follow, and as the three sera have given the same results, in approximately the same dilution, only one set of records will be described.

1. Precipitin Tests.

a. A faint but positive reaction was obtained with washed placental extract (0.05 c. c.) and the immune sera in dilutions of from 1 : 10 to 1 : 20 corresponding to 0.2 to 0.1 c.c. *Normal rabbit serum gave no precipitate to placental extract or to the other substances tested in Experiments e to h.*

b. The cord blood, from two cases, gave similarly positive results.

c. Retro-placental blood serum, from two cases, gave a distinct but weaker reaction.

d. Normal human blood serum (from a male) gave a precipitate, in the same dilutions as in Experiment *a*.

e. Partial precipitates were sought for. To the immune serum was added an excess of normal human serum. After the precipitate had formed, the fluid was cleared by centrifuging, and then placental extract added as in Experiment *a*. No precipitate appeared in the tubes to which placental extract had been added, nor in those into which more human serum was placed, although even stronger solutions than in Experiment *a* were used. After it was observed that the antisera were hemolytic, another method for obtaining a partial reaction was tried. An excess of a five per cent. solution of human corpuscles was added to the antisera and kept in the ice chest for twenty-four hours. To the clear solution was added placental extract and human serum, in the same proportions as in Experiment *a*. No precipitate was obtained. The control with more human blood cells showed no further hemolysis.

f. The urines of six gravid women (containing no albumin) were tested, using 0.1 to 0.05 c.c. of urine, to the same quantity of antiserum as in the other experiments. No precipitate resulted.

g. The urines of two gravid women (containing much albumin, one in the pre-eclamptic stage, the other toxemic) were tested. The one containing the most albumin gave a negative, the other a faint but positive reaction.

h. The urine of a man containing much albumin gave a faint but positive reaction.

The precipitin reaction in all these experiments showed a faint but unmistakable *human reaction, but no specific placental reaction.*

2. Tests for Deflection of Complement.

For experimental details see heading "Nucleo-Proteid Tests."

a. For all three sera it was found that with from 0.004 to 0.001 c.c. of antiserum, and 0.01 c.c. of placental extract, using slightly more than the minimum amount of complement required (0.03 to 0.025 c.c.), hemolysis was incomplete or absent.

b. With human serum (male) a positive reaction was obtained in much greater dilutions (down to 0.001 c.c. of human serum with 0.003 to 0.001 c.c. of antiserum).

Deflection of complement was more strongly marked in the case of normal blood serum than in that of placental extract.

3. Agglutination Tests. Agglutination of a five per cent. solution of normal human blood corpuscles was obtained in dilutions of the antisera between 1:6 and 1:12, 0.5 c.c. of corpuscles being used. Normal rabbits' serum failed to agglutinate.

4. Hemolysis Tests. Hemolysis of 0.5 c.c. of a five per cent. solution of human corpuscles was obtained in dilutions of from 1:5 to 1:12 of the antisera, the controls with normal rabbits' serum again proving negative.

5. Cytolysis Tests.

a. Suspensions of placental cells, obtained by the method used by Flexner and Noguchi (29) in their cytolytic experiments, were added to 0.5 c.c. of the undiluted antisera, to normal rabbits' serum and to normal sodium chloride solution, kept in the incubator at body temperature for one hour, and then in the ice chest for twelve hours. Unstained and stained specimens of the cells were examined. No very marked differences were noted. On the whole the specimens kept in normal rabbit serum showed the best preservation. In all the others the chromatin network was found more coarsely granular and the nuclear outline less distinct; while in the unincubated specimens, made for control, immediately after preparing the cell emulsion, there were numerous Langhans' cells in addition to the syncytium; in the incubated specimens the syncytial cell complexes greatly preponderated.

b. A similar set of experiments to the foregoing were performed, using small pieces of placental tissue instead of isolated cells. All the specimens were transferred to a five per cent. solution of formalin embedded by the celloidin method, cut, and stained with hematoxylon and eosin. No differences were noted except that as in the previous series the specimens preserved in sodium chloride solution showed less perfect staining qualities. Otherwise the placental tissue was perfectly normal.

CONCLUSIONS.

The injection of rabbits' placenta into rabbits produces no isoprecipitins.

From the incomplete experiments performed it would appear that placental injections into animals of the same species cause no changes in the generative organs. Further research into this question will be pursued.

The injection of human placental nucleo-proteid, prepared from placental tissue made nearly blood free, does not produce an antiserum. This result confirms the conclusions of Pearce and Jack-

son, that nucleo-proteids act merely as mild toxic agents, without specific qualities.

The injection, into rabbits, of human placental tissue, rendered practically blood-free, fails to produce any specific reaction. This confirms the view that the serum reaction following the injection of cells into a foreign organism is largely due to the blood contained in the injected tissues.

The injection into rabbits, of the human placenta, made nearly blood-free, produces a weak "human reaction" which can be demonstrated by the reactions for precipitin, deflection of complement, agglutinin, and hemolysis. *No specific placental reaction can be shown.* This is in strict accord with the view that cytotoxines are not specific; that there is no morphological specificity.

The anti-sera obtained showed no cytolytic action; therefore no specific syncytiolytic action could be demonstrated.

If the information obtained in this investigation is applied to the theory of Halban, it will be noted that no experimental proof of the specific action of placental tissue upon the female generative organs could be demonstrated. The number of experiments performed, bearing upon this one point, were however too few to permit of a definite and final opinion.

The work dealing with Veit's ingenious hypothesis was more complete and carried out by many methods, which would necessarily act as a check upon one another. As the results of all these experiments were in complete harmony, I feel justified in making a positive statement that *no experimental proof of a specific placental immune reaction can be demonstrated by our present biological methods.* Whether Veit's hypothesis, thus deprived of its biological proof, must in consequence be discarded, is a question which I do not consider myself competent to answer.

In conclusion I desire to thank Dr. Simon Flexner, Director of the Rockefeller Institute for Medical Research, for extending to me the privileges of his laboratories. I also wish to acknowledge my indebtedness to Drs. Jobling, Noguchi and Levene for the frequent advice and assistance I have received from them.

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THE NATURE OF THE ANTITETANIC ACTION OF EOSIN.

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In a previous publication[1] Dr. Flexner and I gave the results which we obtained from a study of experimental tetanus in rats and guinea pigs subjected to the influence of certain photodynamic anilines. To recall briefly the main facts brought out there, it may be stated that a solution of eosin, of a certain concentration, destroys in vitro the haemolytic and tetanospastic principles of tetanus toxin, and when applied to the site of inoculation of tetanus spores on threads, or tetanus-toxin, either entirely prevents the appearance of the tetanic symptoms, or delays the appearance and diminishes the effect, and, in rats, sometimes prevents the fatal issue. In a great number of instances in which the eosin is applied directly to the spore-infected area in rats, the lives of the animals can be saved. The next step in this investigation was the determination of the mode of action of the eosin through which the effects already described are produced.

The present paper deals with the manner of action of eosin upon *Bacillus tetani* without and within the body of animals. The subject falls naturally, therefore, into the two main divisions of the action of eosin upon the tetanus bacillus *in vitro* and *in vivo*.

THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VITRO.

Relating to this topic, the following points were considered: (1) the influence of eosin upon the germination, growth, sporulation, morphology, and toxin production of *B. tetani*; (2) the bactericidal and sporicidal effects of eosin upon *B. tetani*; (3) the viability of *B. tetani* in eosinized media; (4) the toxin-producing power of *B. tetani* exposed to the action of eosin.

[1] Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.

Cultivation of Bacillus tetani in Eosinized Culture Media. (*a*) *Eosinized Glucose Bouillon.*—Two kinds of eosin—"rein" and "gelb"—were employed in strength of 0.001 per cent., 0.01 per cent., 0.1 per cent., and 1 per cent. A vigorous culture of the tetanus bacillus was inoculated and incubated at 37° C. in an atmosphere of hydrogen for 10 days.

The control cultures were abundant; they contained typical bacilli forming few threads and showing few spores. The growth in 0.001 per cent. eosin was equal to the control, but microscopically the thread forms predominated and no spores whatever could be found. The growth in 0.01 per cent. eosin was equal to the control, but upon microscopic examination only asporogenous thread forms were seen. The growth in 0.1 per cent. eosin was slight and showed single, asporogenous bacilli. No growth whatever took place in the 1 per cent. eosin medium.

The first effect which the eosin produces, apparently, is to suppress sporulation of the bacilli, and the next is to increase thread formation. The limit of concentration of eosin which permits restricted germination is between 0.1 and 1 per cent., and is probably not far from the former concentration. A later determination placed this limit at 0.2 per cent. The cultures prepared as described were filtered through porcelain and tested for toxicity upon rats. The number of m. l. d. per cubic centimeter of filtrate was as follows:

Control = 1000 m.l.d.			
0.001	per cent.	eosin = 700	m.l.d.
0.01	per cent.	eosin = 100	m.l.d.
0.1	per cent.	eosin = 1	m.l.d.
1.0	per cent.	eosin = 0	m.l.d.

No remarkable difference in the properties of the two kinds of dye was noticed. The reduction in toxicity is greater than the reduction in growth caused by the eosin. The diminished toxicity has, doubtless, a two-fold origin: it arises from the diminished multiplication of the bacillus, and from the injury exerted by the eosin upon the toxin originally produced. The degree of this destruction can be deduced from the relative strengths of the filtrates from the control and the 0.001 per cent. eosin cultures, and it amounts to about 30 per cent. As the eosin strength grows, the effects upon the multiplication and toxicity increase, but not in equal ratio. A corresponding comparison of the hæmolysin (tetan-

olysin) present in the several filtrates was also made. Each cubic centimeter contained the following units:

Control filtrate	contained	*C.H.D.	20	*M.H.D.	333
0.001	per cent.	"	"rein"	"	"	12	"	242
0.001	"	"	"gelb"	"	"	10	"	200
0.01	"	"	"rein"	"	"	4	"	66
0.01	"	"	"gelb"	"	"	3	"	50
0.1	"	"	"rein"	"	"	0	"	0
0.1	"	"	"gelb"	"	"	0	"	0

* C.H.D. = complete hæmolytic dose; M.H.D. = minimal (trace) hæmolytic dose.

Eosin destroys or suppresses the tetanolysin in the cultures as it does the tetanospasmin, a fact which could be predicted on the basis of the observations of Flexner and Noguchi.[2] Note should be taken of the greater activity in this respect of eosin "gelb" as compared with eosin "rein."

(*b*) *Eosinized Glucose Agar.*—The advantage for the study of *B. tetani* which glucose agar has over glucose bouillon is derived from the fact that in the former medium sporulation is much more abundant, and is already easily noticeable after twenty-four hours growth. At the end of seven days spores are numerous, and at the expiration of about thirty days, no vegetative bacilli remain. Hence, to glucose agar the following strengths of both eosins were added, 0.01 per cent., 0.02 per cent., 0.05 per cent., 0.1 per cent., 0.2 per cent., 0.5 per cent., and 1 per cent. Stab inoculations were made; cultivation at 37° C.

The growths were well-marked in the tubes below 0.05 per cent. strength; above this limit the growths diminished progressively with increase in concentration. On microscopical examination, it was found that 0.1 per cent. of eosin was the limit of concentration for spore formation. Below this concentration, sporulation took place; at and above it, no sporulation occurred even after three months. The number of spores in eosinized media is below the number in ordinary media; the situation of the spores in the former media is the centre of the body. Transplantation of the atypical sporulating bacilli to simple glucose agar brings about immediate

[2] *Op. cit.*

return to typical mode of sporulation. The limit of germination is for eosin " rein " about 0.5 per cent., while for complete germination, it is about 0.2 per cent. Strengths above 0.5 per cent. inhibit vegetation of spores, but they do not kill the spores. Eosin "gelb" is somewhat more inhibitory. Concentrations above 0.02 per cent. prevent sporulation, but permit vegetation; above 0.5 per cent. no vegetation occurs.

(*c*) *Eosinized Tissue Bouillon.*[3]—The tetanus bacillus grown aerobically at 37° C. in tissue bouillon produces abundant spores in a few days. The sediment which forms in the cultures in a few days, is composed largely of spores. The effect of eosin " gelb " on this process was studied by adding strengths of 0.001 per cent., 0.003 per cent., 0.01 per cent., 0.03 per cent., 0.1 per cent., 0.3 per cent., 1 per cent., and 2 per cent. of the dye. The tissue absorbs a part of the dye; the cultures were incubated at 37° C. for twenty hours when first examined, and subsequently for twenty-nine days longer.

After 20 Hours.		
Control.	Good growth; gas formation.	C.S.* single bacilli; no spores.
Eosin "gelb" 0.001%	Idem.	C.S. chiefly single, few threads, no spores.
" " 0.003	Idem.	C.S. bacilli chiefly in chains, no spores.
" " 0.01	Less growth.	C.S. long threads only showing vacuoles and irregular contours.
" " 0.03	Idem.	Idem.
" " 0.1	Slight growth.	C.S. very few single bacilli, small number of spores.
" " 0.3	Doubtful growth.	C.S. few bacilli and spores.
" " 1	No growth.	C.S. very few spores.
" " 2	Idem.	Idem.
After 30 Days.		
Control.	Fluid clear; deposit heavy.	C.S. all bacilli bear spores.
Eosin "gelb" 0.001%	Idem.	C.S. numerous threads and spores.
" " 0.003	Idem.	C.S. nearly exclusively in threads; very few spores.
" " 0.01	Idem.	C.S. threads; no spores.
" " 0.03	Idem.	Idem.
" " 0.1	Slight deposit.	C.S. a few short chains; many single bacilli; few spores.
" " 0.3	No change.	
" " 1	No change.	
" " 2	No change.	

* C.S. indicates cover slip preparations stained in the usual way.

[3] Theobald Smith, *Jour. of the Boston Soc. of the Med. Sciences,* 1901, iii, 340. Tarozzi, *Centralbl. f. Bakt.,* etc., Orig., 1905, xxxviii, 619.

The foregoing observations are in conformity with those already recorded. In spite of the vigorous growth of the bacilli in this medium containing small quantities of eosin, the biology of the organisms is influenced directly as regards their power to segment and to form spores. With increasing concentrations of the eosin these effects become more pronounced, until in the concentration of 0.1 per cent. only part of the transplanted spores are enabled to germinate, and the vegetative bacilli produced by this imperfect germination are restrained from free multiplication. This process of imperfect vegetation comes, I believe, to play a very interesting part in developing immunity in inoculated rats treated with eosin, of which phenomenon I will have occasion to speak hereafter.

(*d*) *Bactericidal and Sporocidal Properties of Eosin upon B. tetani.*—Glucose bouillon cultures of the tetanus bacillus do not form spores within forty-eight hours, hence such cultures can be employed to test the bactericidal effect of strong solutions of eosin. It was found that such spore-free cultures when mixed with eosin to the concentration of 2 per cent., and kept for fifteen minutes in diffuse light, fail to grow upon replantation. If the eosin strength falls below 1 per cent., not all the bacilli are killed. Contact for twenty-four hours of vegetative sporeless bacilli with eosin in solutions above 0.1 per cent., causes their death. The bactericidal effect of eosin is increased by exposure to the sun: eosin in a strength of 0.02 per cent. can cause in eight hours the death of vegetating bacilli, when exposed directly to the sun's rays. If the experiments are made under anaerobic conditions the results are not essentially different.

The spores of the tetanus bacillus display far greater resistance. Solutions of eosin of 5 per cent. and 0.05 per cent. failed to bring about their destruction after exposure under aerobic and anaerobic conditions to the direct rays of the sun for thirty hours.

(*e*) *Viability of B. tetani in Eosinized Media.*—Cultures eighty-eight days old of the tetanus bacillus in glucose agar containing 0.1 per cent. eosin "gelb" showed no spores. On transplanting from these cultures into glucose agar, a feeble growth containing few spores after seven days was secured. Transplantations into esoin-free media from this slight growth gave cultures which be-

haved, in all respects, in a normal manner. From this experiment the conclusion can be drawn that concentrations of eosin which are not quickly fatal to sporeless tetanus bacilli reduce in the first generation their power of reproduction but do not suffice to kill them outright even after long periods of contact.

(*f*) *Can B. tetanus be Rendered Durably Asporogenous by Eosin?*—Eosin in the strength of 0.01 per cent. reduces spore-formation in *B. tetani* and in the strength of 0.1 per cent. prevents it entirely. A strain of *B. tetanus* was cultivated in 0.01 per cent. and 0.1 per cent. successively through many generations. At each transfer to eosinized medium a control culture was made in glucose-agar to observe the point of final disappearance of the spore-bearing faculty. In the case of eosin "rein," the bacillus withstood fairly well the successive implantations into the eosinized medium, but the faculty to produce spores in ordinary media was not lost after eight generations, covering a period of three months, in eosinized glucose agar. A reduction of the spore-formation was noted in the first generations in the plain glucose medium. In the case of eosin "gelb," the bacillus survived for three generations only in 0.1 per cent. eosin-glucose-agar, and for the eight generations in the 0.01 per cent. medium. No permanent alteration of spore-bearing capacity was effected in the latter cultures.

(*g*) *Is the Toxin Producing Power of B. tetani Affected by its Growth in Eosinized Media?*—Two cultures of *B. tetani* were kept in 0.1 per cent. eosin "gelb" glucose-agar for eighty-eight days, after which they were renewed by transplantation to glucose bouillon. After forty-eight hours growth at 37° C., these cultures and suitable control cultures were filtered through porcelain. The toxicity of the filtrates was approximately equal. The deduction from this experiment is obvious: no permanent influence upon the toxin-producing faculty of *B. tetani* is exerted by long contact of eosin in concentrations below the bactericidal limit.

THE ACTION OF EOSIN UPON THE TETANUS BACILLUS IN VIVO.

The next subject of the study taken up related to the manner in which eosin acts in preventing tetanus in rats inoculated with tetanus bacilli or their spores.[4] The effects to be explained are,

[4] Flexner and Noguchi, *op. cit.*

briefly, these: rats, beneath the skin of which tetanus spore-threads are placed, regularly develop tetanus and die. If, however, the inoculated rats are treated by injections of eosin about the spore-thread, many recover, and some even fail to develop any symptoms of tetanus. Injection of the eosin in other parts of the body may delay the appearance of tetanus and the fatal issue, but does not suffice wholly to prevent them. The effect of the eosin on the local reaction to the tetanus bacilli in the inoculated rats is to be explained.

Fate of Spores Introduced into the Body on Threads.—Eighteen rats (weighing about 90 grams each) were inoculated beneath the skin of the thigh with spore-threads of *B. tetani,* free from toxin, on June 8, 1906. The six of these left untreated (controls) developed tetanus in two to six days time (average, three days). Death usually resulted on the third day after the appearance of the tetanus. Cultures were made from the threads and the liver.[5] Tetanus bacilli were recovered from the threads in all six animals and from the liver in three animals. The remaining twelve rats were treated with eosin "rein." Doses of 0.2 to 0.4 c.c. of a 2 per cent. solution were injected successively on three days, beginning immediately after the inoculation, about the thread, and intermittently (every other day) for three more injections. The treated animals did not develop tetanus, and a part of them were alive and healthy four months after the inoculation.

The first question which I asked myself is, *Do the tetanus spores germinate and multiply under the influence of the eosin, and what becomes of the germinated bacilli?*

Experiment. Rat No. 7. A part of the spore-thread was removed forty-eight hours after inoculation and during the eosin treatment. Cover slips prepared from it showed many spores, a small number of bacilli and a few leucocytes. A second portion of the thread was removed on the ninth day (day of last eosin injection). Cover slips showed very few bacilli and many spores and leucocytes. Cultures gave pure growths of *B. tetani.* On the thirteenth day, the remainder of the thread was removed. Now only spores could be found, and leucocytes were no longer present. The rat remained well.

Rat No. 8. Same treatment; examination on fifth day; among many spores a small number of bacilli—vegetative forms—were seen.

[5] Tarozzi, *Centralbl. f. Bakt.,* etc., 1906, xl, 305, 451.

The study of the spore threads in the two inoculated rats show conclusively that the injections of eosin do not wholly prevent the germination of the spores, but that the germination is largely suppressed; and it also renders the view very probable that of the germinated spores few or none multiply in the eosinized tissues, while after a short time (few days) the vegetated bacilli themselves disappear. Attention is called to the observation that the tetanus spores remain in an intact state for many days in the inoculated region of the body.

The next question which I asked myself is, *What becomes eventually of the ungerminated spores, and, supposing they remain locally in the tissue, do they suffer any alteration in toxin-producing power?*

Experiment. Rat No. 9. Inoculated June 8. Eosin-treated. No symptoms, June 24. Removed the spore-thread which was found to be surrounded by fibrous tissue and adherent to the fascia. Eosin had disappeared. Cover slips showed many spores and no bacilli. A pure culture of *B. tetani* obtained in tissue bouillon.

Rat No. 10. The thread was removed nineteen days after inoculation. The result was in all respects like that of rat No. 9. Toxin of the usual strength was yielded by the cultures.

Rat No. 11. A part of the spore-thread was removed twenty-three days after the inoculation. Cover slips showed many spores which yielded a pure culture of *B. tetani* containing a strong toxin. The second wound healed without giving rise to tetanus. Animal remained well.

By these observations it is shown that the healing of the local wound containing the eosinized threads, proceeds in a manner similar to that of a sterile wound; and it is also shown that, by virtue of the eosin treatment, the tetanus spores are reduced to the value of inocuous foreign bodies. In the course of this process, the tetanus spores, already quiescent, remain in the healed tissues for an indefinite time. They are not devitalized by the tissues, or, apparently, altered in any essential way, at least, their toxin-producing power is not impaired by the new conditions under which they survive.

If the results obtained in vitro and in vivo are compared certain correspondences, as well as certain differences, can be noted. Perhaps the most important difference consists in the disappearance of the vegetative bacilli in the body, and their persistence in the test tubes, in the tests with eosin; and the germination of the re-

strained spores in the culture once the eosin is no longer present, while, in the body, the tissues already advanced in healing restrain the vegetative propensities of the spores, although the toxin has been entirely eliminated.

The healing of the wound of the second operation in rat No. 11, without giving rise to tetanus, all the eosin having meanwhile disappeared, is an interesting fact, and one that deserves further consideration. The cultivation experiment proved that living tetanus spores, capable of toxin-production remained in the thread; and yet the remainder of the thread, in the tissues, gave rise to no symptoms. It might be thought indeed, that the spore threads, after this long sojourn in the body, are not capable of directly infecting a second animal. A test of this possibility was easily made.

Experiment. Rat No. 12. A normal rat was inoculated on July 9 beneath the skin of the thigh with a spore-thread removed on the thirty-second day from an eosin-treated rat. On July 11 the animal was tetanic, and on July 13 it was dead. A pure culture of *B. tetani* was recovered from the thread.

The apparent innocuousness of the second operation for the removal of spore-threads in animals treated long before with eosin, and the great susceptibility of the normal rat to the threads after this long residence in the body, brought up the question of the possible existence of a local immunity to tetanus in the eosin-treated animals. This idea was, of course, capable of experimental verification. The experiments relating to it form the subject of a separate publication.

SUMMARY.

Eosin, if present in cultures containing tetanus spores, prevents the germination of these spores when its concentration (in glucose bouillon) reaches 0.2 per cent. When the concentration of the eosin sinks to 0.01 per cent., germination of the spores is no longer inhibited, but the vegetative bacilli developed from the spores execute a highly restrained form of multiplication. When the eosin concentration sinks to 0.001 per cent., vegetation and multiplication of the bacilli become more active, but no new spores are formed even after long periods of time. With glucose agar it is not until the concentration of the eosin in the cultures falls to 0.05 per cent. that sporulation again appears. At this concentration of the eosin,

very few spores are formed; but as the eosin sinks lower and lower, sporulation becomes more active, until with 0.001 per cent. it is essentially of normal degree. In concentrations of 0.003 per cent., eosin prevents perfect segmentation of the multiplying bacilli, with the result that, finally, long and convoluted threads of bacilli are produced. The spores which are formed in a medium containing 0.01 per cent. of eosin are often situated at the centre and not at one pole of the bacilli.

Eosin in a strength of 2 per cent. is capable of destroying the vegetative bacilli, if the contact is prolonged to fifteen minutes, and in strength of 0.1 per cent., in twenty-four hours. Placing this latter mixture of bacilli and eosin in the sunlight greatly hastens the bactericidal effect, and the bacilli are found to be incapable of growth at the end of several hours. Eosin in high concentrations is not capable of killing the tetanus spores, even after long exposure to sunlight (thirty hours).

The toxin production of tetanus bacilli grown in eosinized culture media diminishes as the concentration of the eosin increases. This effect is brought about partly by the restraining action of the dye on vegetation, and partly by its detoxicating action upon the poison.

The toxin-producing power and the virulence of tetanus bacilli are not permanently modified by contact with eosin for a long period, or by successive cultivations in eosinized media.

Eosin is likewise capable of restraining the vegetation of tetanus spores in the animal body. In spore threads inserted beneath the skin of rats, and surrounded with eosin in solution, a very restricted vegetation takes place. If the injections of eosin are repeated, vegetation soon ceases and the vegetated bacilli degenerate and disappear.

The ungerminated tetanus spores remain alive in a latent condition indefinitely in the healed wound beneath the skin. These spores do not lose power to grow outside the body, or inside the body of animals under favorable conditions, or to produce toxin in a characteristic manner.

LOCAL IMMUNITY TO TETANUS IN INOCULATED RATS TREATED WITH EOSIN.

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Previous studies having shown the mechanism of the immunity to tetanus which develops in rats treated locally with eosin, my attention was directed by Dr. Flexner to certain indications of the occurrence in the treated animals of a local in contrast to a general immunity to the tetanus bacillus. Our studies[1] had already shown that the spores on silk threads in the healed eosin-treated wounds remain alive for an indefinite period, and the threads removed and transplanted to other rats, or to the opposite side of the body of the same rat, caused fatal tetanus. And yet, the operation for the removal of part of the spore-threads was not followed, in spite of the trauma inflicted and the portion of the spore-thread which remained, by any tentanus in the extremity originally infected. Since it appeared that the conditions were, after the second operation, similar on both sides of the body, or in the two animals, an explanation of the different reactions observed was required. It seemed natural to assume, for the sake of further experiment, that the restricted germination and vegetation which take place in the eosin-treated animals produce a small quantity of tetanus-toxin, which, acting locally upon the tissues, gives rise in them to a degree of immunity to the action of the tetanus poison.[2]

The observations upon which this hypothesis was first based will be given; but it should be stated in advance that the tetanus-bearing threads were introduced subcutaneously in the thigh and not deeply into the tissues. After the healing process is complete, the threads come to lie in a small mass of scar tissue between the superficial

[1] Flexner and Noguchi, *Jour. of Exper. Med.*, 1906, viii, 1.

[2] Noguchi, "The Nature of the Antitetanic Action of Eosin," this number of the JOURNAL.

fascia and the skin. Except for the cicatrix the tissues all return apparently to the normal condition.

Experiment I.—In order to determine whether the spores remained alive in the inoculated rats after the healing process was complete, a portion of a thread was removed thirty days after inoculation and fifteen days after the cessation of the eosin treatment; no trace of the eosin remained at this time in the tissues. The removed portion of the thread was used for preparing cultures and for inoculating rat No. 12. Tetanus bacilli grew in the cultures. Tetanus developed in rat No. 12 on the second day, and death from tetanus took place on the fourth day after inoculation. The treated and operated rat remained well.

Before discussing further the significance of this experiment, a selected series of protocols bearing on this question of local immunity to tetanus will be first given.

Experiment II. Rat No. 13.—Thirty days after inoculation and fifteen days after cessation of eosin treatment, a portion of the healed-in spore-thread was removed from the right thigh. No tetanus followed the trauma, and the wound quickly healed. Six days later, a second portion of the thread was removed and implanted subcutaneously under the skin of the left thigh. Tetanus developed on the left (new) side on the second day, and death occurred on the fourth day after this inoculation. The right (original) hind leg remained entirely free from tetanus.

Experiment III. Rat No. 14.—Eosin treatment. Seventeen days after inoculation the thread was removed from the right thigh; the wound of the second operation was completely healed in seven days, during which period no tetanus developed. July 12, reinoculation of spore-threads beneath the skin of both thighs. July 16, left leg tetanic; right leg free. July 18, tetanus has progressed on left side; right side free. July 19, rat dead. The animal lived three days after the first appearance of tetanus. The right (old inoculated) leg remained free from tetanus until the end.

Experiment IV. Rat No. 15.—Spore-thread inoculation into right leg June 8; eosin treatment until June 20. No tetanus. Spore-thread removed July 28; no tetanus. October 9 (123 days after inoculation, and 111 days after last injection of eosin), 0.00008 c.c. tetanus toxin injected into each thigh (0.0003 = m.l.d. in three days). Tetanus developed in the left leg in forty hours, and advanced rapidly; slight tetanus developed in the right leg after three days, and advanced slowly. Death on seventh day.

Experiment V. Rat No. 16.—Spore-thread inoculation into right leg October 29; eosin rein treatment until November 8. No tetanus. December 1 (33 days after inoculation and 25 days after last injection of eosin) 0.0004 c.c. tetanus toxin injected into each thigh. Tetanus developed in left leg after three days, and advanced rapidly; a mild form of tetanus developed in the right leg and advanced slowly. Both sides became strongly contracted; death on sixteenth day.

Experiment VI. Rat No. 17.—Spore-thread inoculation into right thigh October 19. Eosin gelb injection until November 2. No tetanus developed. December 1 (43 days after inoculation and 29 days after last injection of eosin),

0.0004 c.c. of tetanus toxin injected into each thigh. No tetanus developed in five days. Second injection into each thigh of 0.0004 c.c. toxin. Next day tetanus began in left leg; none in right leg. The tetanus advanced in the left and remained absent from the right side until death, which took place on the ninth day.

Experiment VI brings out clearly, for the first time, the existence of a degree of general immunity to the toxin which is, however, able to off-set a small and limited amount only of the effects of the toxin. The local immunity of the right thigh proved, upon a second injection of the toxin, to be still effective, while the general immunity, through which the left leg had been protected from the consequences of the first injection, was exhausted by it. The next experiment indicates that even a higher degree of general immunity may arise from the local infection.

Experiment VII. Rat No. 18.—Spore-thread inoculation into right thigh; eosin treatment. No tetanus developed. October 9 (123 days after inoculation and 111 days after last injection of easin), 0.00008 c.c. tetanus toxin (0.00008 = m.l.d.) injected into each thigh. No tetanus after three days. October 12, 0.00004 c.c. toxin injected into each leg. October 19, no tetanus developed. The spore-thread from this animal transplanted to another rat produced typical tetanic symptoms, followed by death in two and a half days.

Experiment VII is a stronger confirmation even than experiment VI of the idea that general immunity to tetanus toxin may be developed in the eosin-treated rats. The degree of general immunity is, in almost all the rats, lower than that of the local immunity. Rat No. 18 seems to supply the one exception to what appeared as the rule in this respect. It was now desirable to ascertain by a direct experiment whether the blood of the eosin-treated rats contained a measurable quantity of tetanus antitoxin.

Three series of rats were taken: (1) normal rats as controls; (2) rats which after the spore-thread insertion had been treated with eosin "gelb;" (3) rats which after inoculation had been treated with eosin "rein." The rats were bled from the carotid artery, and the serum collected. On account of the small amount of blood yielded by these animals, the blood of the several animals of each series was collected together. The eosin-treated rats were bled from sixty-four to seventy-four days after the cessation of the eosin treatment. The following tabulation gives the results of the tests. In each instance, the mixtures of serum and tetanus

toxin were kept at 37° C. for half an hour before injection. The injections were made beneath the skin of the thigh.

Serum Injected.	Toxin Injected.	Tetanus Developed.	Result.
Normal 0.3 c.c.	0.006 c.c. (6 m.l.d.)	24 hours.	Death on 3d day.
Normal 0.3 c.c.	0.002 c.c. (2 m.l.d.)	24 hours.	Death on 6th day.
Saline 0.3 c.c.	0.006 c.c. (6 m.l.d.)	24 hours.	Death on 3d day.
Saline 0.3 c.c.	0.002 c.c. (2 m.l.d.)	24 hours.	Death on 5th day.
Eosin gelb "a" 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Tetanus increased but did not extend beyond injected leg. 28 days after injection still alive; tetanus gradually disappearing.
Eosin gelb "b" 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Tetanus progressed, death on 9th day.
Eosin rein 0.3 c.c.	0.006 c.c. (6 m.l.d.)	slightly in 48 hours.	Death on 10th day.
Eosin rein 0.3 c.c.	0.002 c.c. (2 m.l.d.)	5th day.	Death on 15th day.

The experiments which I have set down in this article seem to me to afford convincing proof of the existence of local immunity to tetanus infection and tetanus intoxication. The observation is, I believe, new and not without interest in its bearing on the theory of the "building sites" of immune bodies in the animal organism. Examinations of the lesion consisting of the scar enclosing the spore-threads have shown it to be superficial, limited to the fascia and adjacent subcutaneous tissue, and to measure a few millimeters in extent only. There are no anatomical changes discoverable which would connect the lesion with the deeper muscles, fascia, nerves, etc.

From the studies made of the spore-threads at different periods during the eosin treatment,[3] a complete idea of the evolutions through which the introduced spores pass has been obtained. A certain small number of the spores germinate and grow into vegetative bacilli, which, after having reached this stage of development, remain as such for a time, or, possibly, pass through a small series of divisions with the production of a few generations of bacilli. The number of divisions is at best few, and the total number of bacilli produced from the spores is relatively small. The vegetative bacilli degenerate within a few days, and the majority of

[3] Noguchi, "The Nature of the Antitetanic Action of Eosin," this number of the JOURNAL.

the spores do not germinate at all, but remaining alive and quiescent, are enclosed in the scar tissue.

Doubtless, there is associated with the low degree of vegetation of the tetanus bacilli toxin production. This toxin, always small in amount, is held chiefly in or near the locality in which it is formed by the surrounding wall of eosin through which it must, in the first place, pass slowly and with difficulty, and which, in the next place, would tend to detoxicate it on its passage. The effect, therefore, of the toxin would be exerted locally, if anywhere, and if any cells capable of being excited to antibody-production exist in the situation in which the toxin finds itself, they, presumably would be stimulated into activity. Should any of the toxin, in a still active state, diffuse beyond the eosin barrier, it would find itself in places from which it could readily pass into the blood and into the internal organs, where it would be exposed to the usual conditions of antibody formation. I conceive that the mechanism of the antibody production in the particular case brought out by these experiments is to be explained somewhat in the manner of this supposition.

The questions immediately arise as to which cells in the local tissues are concerned with the elaboration of the antitoxin, and whether the antoxin as such is stored in these cells for immediate liberation when called forth by the presence of the specific antigen. As regards the first question, no definitive answer can be given. From the circumstances of the experiment, I am led to suppose the connective tissue cells, possibly the endothelium of the lymphatics as well, as the cells yielding the antibody. The extremely superficial character of the lesions must be kept in mind in attempting to fix the parts the cells of which participate actively in the production of this local immunity. But I cannot bring forth proof that the active cells are so strictly limited and so sharply circumscribed as I have indicated. It would, theoretically, seem to be less difficult to supply an answer to the second question. It does not seem to me highly likely that so diffusible a substance as antitoxin in the free state could remain in a state of high concentration through many months in one part of the body at the same time that it existed in very low concentration elsewhere in the body.

On the contrary, I should be more inclined to the view that a large part of the general antitoxic immunity which the rats exhibited may have been derived from the antibody absorbed from its local site of production. The cells of the locally immune tissues have, I assume, undergone a physiological change which endures for many months, at least, enabling them to withstand the injurious effects of tetanus bacilli and tetanus toxin either by means of rapid liberation of antitoxic substances, or by an increased form of resistance to and destruction of poison (Giftfestigkeit) and bacilli with which the liberation of antagonistic antibodies is not necessarily associated. Upon what remarkable changes in function or structure this power depends, we are in total ignorance, and any speculation must, therefore be wholly hypothetical.

The literature on immunity contains, as is well-known, other examples of local immunity. Doubtless in every case, at some period of antibody production, there exists a local immunity which exceeds in degree that which the blood is able to display. This fact must be acknowledged by all who believe antibodies not to be formed chiefly in the blood itself. It will suffice merely to refer to the observations of Pfeiffer and Marx[4] on the organs in which the antibodies to the cholera bacillus are produced; to the experiments of Wassermann and Takaki[5] of the fixation of tetanus toxin by the brain and other organic tissues; to the studies of Wassermann and Citron[6] on the local production by the pleural and peritoneal endothelium of antibodies (bacteriolytic, possibly others also) for the typhoid bacillus; to the interesting experiment of von Dungern[7] on the local production of precipitin for Maja serum in the anterior chamber of the eye; and, finally, to the observations of opthalmologists[8] upon the local immunity developed by the conjunctiva to abrin inoculation, a phenomenon which Römer studied with great care and precision. Theoretically, there is much similarity in all these observations, since they prove that anti-

[4] *Zeit. f. Hygiene und Infektionskrank.*, 1898, xxvii, 272.
[5] *Berl. klin. Woch.*, 1898, xxxv, 5.
[6] *Zeit. f. Hygiene und Infektionskrank.*, 1905, l, 331.
[7] Die Antikorper, Jena, 1903.
[8] Von Hippel, *Archiv f. Ophthalmolog.*, 1883, xxix, 213. Sattler, *Klin. Monatsbl. f. Augenheilkr.*, 1883, xxi, 207. Neisser, *Fortsch. d. Medicine*, 1884, ii, 73. Römer, *Archiv f. Opthalmologie*, 1901, lii, 172.

bodies are produced locally. The gradually increasing number of facts relating to this subject tend to exalt in importance cells which hitherto have been regarded as indifferent in respect to antibody production: namely, the cells of the connective tissues, lymph spaces, lymph vessels and serous cavities. The particular observations which form the basis of this paper show that the antibodies to the tetanus poison can be produced in quantity by other cells than those of the central nervous system, for which, apparently, tetanus toxin has an especial affinity.

PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES OF MAGNESIUM SALTS.[1]

V. The Influence of Nephrectomy upon their Toxicity.

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Introductory.—The narcotic effect as well as the toxicity of magnesium salts, when introduced subcutaneously, depend to a great degree upon the readiness with which they are eliminated from the body. By what path are these salts eliminated? Are they excreted by the intestinal canal or are they eliminated through the kidneys? Meltzer and Auer[2] state that from their experiments they gained the impression " that animals which urinated frequently had a better chance for recovery. Early urination seemed also to prevent the full development of anesthesia. Furthermore it seems that magnesium sulphate after subcutaneous injection acted as a diuretic." Such observations seemed to indicate that the kidneys are taking an essential share in the elimination of the magnesium salts. On the other hand J. B. MacCallum[3] states that magnesium salts (magnesium chloride) not only do not act as diuretics, but exert directly an inhibitory effect upon the urinary secretion. Furthermore from a remark recently made by Mendel it seems to follow that magnesium as an alkali earth leaves the body by the bowel. Mendel[4] states that the studies of the paths of excretion for inorganic compounds seem to " indicate a noteworthy difference in the mode of elimination of these alkali metals in contrast with corresponding salts of the alkali earths. The latter have been shown to leave the body in far greater quantity by the bowel than in the urine."

[1] Studies I–IV were published in the *American Journal of Physiology.*

[2] Meltzer and Auer, *American Journal of Physiology,* 1905, xiv, 386.

[3] MacCallum, "On the Mechanism of the Physiological Action of the Cathartics," Berkeley, 1906, p. 45.

[4] Mendel and Closson, *American Jour. of Physiol.,* 1906, xvi, 45.

Of the alkali earths Mendel himself studied the elimination of strontium[5] and barium salts.[6] For the paths of elimination of calcium salts Mendel refers to Rey.[7] For the salts of these three alkali earths it was variously established experimentally, as stated above, that no matter by what path introduced, they leave the body in far greater quantity by the bowel than in the urine. Magnesium like calcium is an alkali earth and from the rule laid down by Mendel for this class of inorganic compounds it seems to follow that the salts of magnesium also leave the body in far greater quantity by the bowel than in the urine. Are there any experimental facts for such an assumption?

Mendel who does not discuss specially the paths of elimination of magnesium, gives only one reference; it is to Lusk.[8] But here we meet with a statement which is contrary to that assumption. Lusk says that "in carnivorous urine the major part of excreted magnesium is found in the urine, the balance being given off through the intestinal wall to the feces." Lusk mentions no authority for his statement. But the literature on metabolism of inorganic salts contains many studies which bear out this view of Lusk. In the extensive studies of F. Müller,[9] for instance, we find a statement which is exactly like the one made by Lusk. He says "dass beim Fleischfresser der Kalk zum weitaus grössten Theil durch den Darmkanal aus dem Organismus ausgeschieden wird, während die Magnesia . . . hauptsächlich durch den Harn verlässt." Other writers speak of the fraction of magnesium salts which leaves the body through the kidney as being between thirty and forty per cent. However this may be, there is a unanimity of opinion amongst all the investigators, at least as far as we can see, on one point which is of especial interest to us, namely that there is a striking difference between calcium and magnesium with reference to the path of elimination,

[5] Mendel and Thatcher, *American Jour. of Physiol.*, 1904, xi, 5.

[6] Mendel and Sicher, *ibid.*, 1906, xvi, 147.

[7] Rey, *Archiv f. exp. Path. u. Pharm.*, 1895, xxxv, 295. See Rüdell, *ibid.*, 1893, xxxiii, 79.

[8] Lusk, "American Text-Book of Physiology," 2d edition, 1900, Vol. i, p. 527.

[9] Müller, *Zeit für Biol.*, 1884, xx, 334. Heiss, *ibid.*, 1876, xii, 156. Bertram, *ibid.*, 1878, xiv, 335. Renvall, *Skandinavisches Arch. für Physiol.*, 1904, xvi, 94.

the former preferring the alimentary canal, while the latter favors the kidney. In the quoted metabolism experiments the salts of magnesium and calcium were administered by the mouth. As far as we know, there are no experiments on the mode of elimination of magnesium salts when they were given subcutaneously or intravenously. Possibly in the latter cases the elimination through the kidney would prove to be still more favored. At any rate the rule laid down for the elimination of calcium, strontium and barium cannot be applied *a priori* to the elimination of magnesium.

We may therefore claim that there is, to say the least, no experimental evidence based on chemical analysis against the assumption that the kidneys are taking an important share in the elimination of magnesium salts. On the other hand, the above quoted observation of Meltzer and Auer "that animals which urinated frequently had a better chance for recovery" may be taken as an indication in favor of such an assumption. The authors, however, had at that time not made a special study of that question; they speak only of their impressions. Besides, the fact that urination and recovery went hand in hand is not safe evidence that the urination contributed to the recovery. Possibly the recovery takes place through the elimination of magnesium into the intestines and urination occurs on account of the removal of magnesium and in accordance with the above mentioned claim of MacCallum that the presence of magnesium in the blood inhibits urinary secretion; the coincidence of recovery and urination would then mean that recovery is the cause and urination the result and not the contrary.

There is, however, another biological method by which the share of the kidneys in the elimination of the magnesium salts can be systematically studied and which is capable of bringing out reliable results. It is the study of the susceptibility of the animal to the toxic effect of magnesium salts after nephrectomy. Such a series of experiments were carried out by us and the object of this paper is the report of the findings of this research.

THE SUSCEPTIBILITY OF THE ANIMAL TO THE TOXIC EFFECT OF MAGNESIUM AFTER NEPHRECTOMY.

Method.—The experiments of Meltzer and Auer on the anesthetic effect of subcutaneous injection of magnesium salts were made on seven species of animals and with the sulphates as well as with the chlorides. The present experiments were made on rabbits only, and only magnesium sulphate was employed. Under ether anesthesia double nephrectomy was performed and after recovery from the ether anesthesia variable quantities of magnesium sulphate in molecular solution were injected subcutaneously, using approximately the same region for injection in all animals and observing the same conditions. The results of this research will be presented best by quoting a few abbreviated protocols of experiments.

Fatal Dose.—In the following experiment one gram of the salt per kilo animal was injected shortly after nephrectomy.

Experiment 1.—June 19, 1906. A series of three rabbits was used.

A. White rabbit, 2,480 grams. 3 p. m. Both kidneys exposed by lumbar route, pedicles firmly ligated and the greatest part of each kidney removed.

3.35. Rabbit recovered from ether, appears quiet and normal. Injected magnesium sulphate ($\frac{M}{1}$ solution), *one gram per kilo weight.*

4.10. Animal drowsy, chin rests on floor, head to one side, respiration shallow.

4.35. Lying on side, completely relaxed.

4.43. No conjunctival reflex; reacts feebly to probe in nose.

5.05. Respiration very shallow, 36 per minute.

5.15. Respiration stopped, heart still continues to beat. Animal died one hour and forty minutes after the injection.

B. (Nephrectomized control.) Black rabbit, 2,340 grams; appears to be less robust than A.

3.20. Nephrectomized as in A.

4.00. Does not recover from the anesthesia as rapidly as A; trembles (carbolic intoxication?).

4.20. Hops around, rises on hind legs and seems normal.

June 20. Eats cabbage, appears normal.

June 21, 10 a. m. Did not eat at morning feeding, drinks a little water.

12 noon, found dead.

This animal died about 45 hours after nephrectomy.

C. (Magnesium control.) White and black rabbit, 1,625 grams, not operated.

3.30. Injected magnesium sulphate ($\frac{M}{1}$ solution) one gram per kilo weight.

At no time was there the slightest sign of a magnesium effect, the rabbit appearing at all times as normal as before the injection.

In this series one gram of magnesium sulphate per kilo animal weight killed the nephrectomized animal one hour and forty minutes after the injection (two hours and fifteen minutes after the operation), whereas the same dose had not the slightest effect upon the normal rabbit and the nephrectomized control animal which received no magnesium, lived forty-three hours and appeared to be normal until a few hours before its death. From the experiments of Meltzer and Auer it appears that a dose less than 1.75 grams per kilo, when given subcutaneously and without massage is not fatal to the rabbit. For the nephrectomized rabbits a dose of only one gram of the salts per kilo animal proved to be fatal; that means an increase of the susceptibility of the animal to the poisonous effect of the magnesium salt nearly equal to fifty per cent.

Experiment 2.—Series of two rabbits. June 25, 1906.

A. Gray rabbit, 2,500 grams.

2.15 p. m. Double nephrectomy.

4.10. Injected one gram magnesium sulphate per kilo animal.

4.30. Animal shows signs of beginning anesthesia and paralysis. All symptoms gradually increased and the animal died at 7 p. m. about three hours after the injection.

B. Control (Rabbit C from former experiment). Not operated.

June 25, 4.10 p. m. Injected 1.6 magnesium sulphate per kilo animal.

5.30. Animal greatly relaxed, lid reflex much retarded. Anesthesia and paralysis were at no time complete; the animal soon began to show recovery, and next morning appeared to be perfectly well again.

In this experiment again in the nephrectomized rabbit one gram per kilo killed the animal in a few hours after the injection, while in the normal animal 1.6 grams per kilo was even insufficient to produce complete temporary anesthesia.

In these and in other similar experiments it was then found that one gram per kilo, injected two or three hours after nephrectomy, proved to be a fatal dose, the animal becoming first anesthetized and paralyzed a few hours after the injection.

Effect of Late Injection.—The result was different, however, when the injection was not administered until about eighteen hours or more after the nephrectomy. The following protocols will illustrate.

Experiment 3.—June 26, 1906. Rabbit A, weighing 1,992 grams, was nephrectomized at 6.30 p. m. Rabbit B, weighing 1,957 grams, was nephrectomized at 6.50 p. m.

A. June 27, 12.35 p. m. (eighteen hours after nephrectomy). Injected magnesium sulphate one gram per kilo in $\frac{M}{1}$ solution; *slight massage.*

2. Chin rests on floor, animal remains lying on side when turned over; recovers posture when tail is pressed.

4.45. Animal lying stretched out, relaxed, conjunctival reflex slight. Reacts to probe in nose and on pressing tail.

June 28, 11.40 a. m. Animal shows some recovery, occasionally makes an effort to rise; lid reflex retarded, but definitely present; on pressing the tail, the animal moves head and feet and cries feebly.

Animal died at 1.30 p. m., twenty-five hours after injection, forty-three hours after operation.

B. June 27, 12.40 p. m. About eighteen hours after nephrectomy injected magnesium sulphate one gram per kilo in $\frac{M}{1}$ solution; massage, stronger than in A.

1.20. Strongly influenced by the salt. Lies flat, head on floor. Raises head when tail is pinched, lid reflex still present.

4.40. Lid reflex completely gone; no reaction to pressure of tail or to probe in nose.

Animal died at 8 p. m., about seven hours after the injection, twenty-five after nephrectomy.

Both animals were injected about eighteen hours after nephrectomy and in both the injected places were massaged, in *B* a good deal more strongly than in *A*. Animal *A* was never completely under anesthesia, had shown later some slight recovery and lived about twenty-five hours after the injection. Animal *B* was completely under anesthesia and died without recovery about seven hours after the injection.

This last rabbit (*B* of Experiment 3) was the only animal which lived so short a time as seven hours after the injection when the latter was given late after nephrectomy. Some animals lived twenty-four hours and more after the injection, and one rabbit lived as long as sixty-three hours after an injection of one gram of the salts per kilo animal, which was given twenty-four hours after the double nephrectomy. The narcotizing effect was in this case fairly outspoken and the recovery was very slight, the animal remaining for about three days in the semi-paralyzed and semi-stuporous state.

In general it may be stated that the later after the nephrectomy one gram salt per kilo was given, the less fatal it proved to be and also, perhaps, the less complete was the state of depression; the recovery, however, was in all cases but slight.

Anesthetic Dose.—In eight experiments the dose of the salt injected into the rabbits amounted only to 0.8 gram per kilo animal. We shall illustrate the results by one abbreviated protocol.

Experiment 4.—June 21, 1906. Gray rabbit, 1,520 grams.

1 p. m. Double nephrectomy.

1.50. Injected magnesium sulphate 0.6 grams per kilo in $\frac{M}{1}$ solution.

3.50. Lying flat, completely relaxed, respiration very slow. Conjunctival reflex present, but diminished.

4.45. Lying completely relaxed on side, conjunctival reflex gone.

6. No change.

June 22, 1.30 p. m. Improved; able to sit up; conjunctival reflex returned.

June 23, 12 noon. Rabbit died, about forty-six hours after injection.

In this experiment with 0.8 grams per kilo, injected very soon after nephrectomy, the animal was completely anesthetized, the depression lasting about twelve hours followed by a moderate degree of recovery. The animal survived the injection by forty-six hours, death apparently being due solely to the nephrectomy. In other experiments with the same dose and under the same conditions the profound anesthesia lasted sometimes more than twenty

FIG. 1. All four rabbits were nephrectomized about twenty-two hours before the photograph was taken. The two animals lying on the table received two hours after the nephrectomy subcutaneous injections of magnesium sulphate, 0.8 per kilo, and about two hours later were completely under the toxic influence of that salt. They remained for about eighteen hours profoundly anesthetized and paralyzed. At the time when the photograph was taken the rabbit on the right hand side showed a slight recovery. The other two rabbits are (nephrectomized) controls.

hours; in other cases again the anesthesia was sometimes a little less complete and the recovery a trifle better than in the last quoted experiment (see Figure). But in the entire series there was no case without distinct anesthetizing and paralyzing effect and none with a fatal outcome due to the magnesium salts. It is hardly necessary to add that in normal rabbits a dose of 0.8 grams per kilo had not the slightest effect.

In a few experiments a dose of 0.8 grams of the salt per kilo animal was given twenty-four hours after nephrectomy. The following protocol is an illustration.

Experiment 5.—July 2, 1906. Grey rabbit, 1,810 grams, 11.40 a. m. Double nephrectomy, perfect recovery.

July 3, 1.54 p. m., about twenty-six hours after nephrectomy, injected magnesium sulphate 0.8 grams per kilo in $\frac{M}{1}$ solution.

3.00 p. m. Animal relaxed, lid reflex very slight.

4.00. Lid reflex gone; no spontaneous movements and no reaction to pressing tail, etc.

10.00. Condition unchanged.

July 4, 10 a. m. Slightly improved, moves head when probe is put in nose, but no reaction to pinching of tail and no lid reflex.

July 5, 10 a. m. Considerably improved; can almost sit up; lid reflex retarded, but present; moves away on pressing tail.

3 p. m. Recovery still more pronounced. Head erect; movements irregular and jerky.

July 6, 8.30 a. m. Found in a dyspnœic state; the animal died five minutes later, about sixty-seven hours after the injection and about ninety-three hours after the operation.

This animal, although injected twenty-six hours after the nephrectomy, became influenced by the salt very rapidly; the depression was very profound and lasted over twenty-four hours. It survived the injection, however, longer than any other animal and the final recovery was more advanced.

Here again, as in the experiments in which a dose of 0.8 grams of the salt was given soon after the nephrectomy, the extent of the effect of the injection was somewhat variable, but the effect was on the one hand never missing and on the other hand never fatal.

In general it may be said that for a dose of 0.8 gram per kilo of the salt there was not a marked difference between the effects of the injections whether given soon after nephrectomy or twenty-four hours later, and this difference certainly cannot be compared

with the striking differences observed in the experiments with one gram per kilo animal.

Minimum Dose.—We have made seven experiments in which a dose of only 0.6 gram of the magnesium salt per kilo rabbit was injected. The following few abbreviated protocols will illustrate the corresponding results.

Experiment 6.—June 21, 1906. Gray rabbit, 1,520 grams.

1 p. m. Double nephrectomy. Completely recovered.

3.20. Injected 0.6 gram of magnesium sulphate per kilo animal in $\frac{M}{1}$ solution.

4.45 and 6 p. m. Appears normal in every way. Animal remained normal until its death which occurred June 24, 8 p. m., about seventy-two hours after injection and seventy-four hours after operation.

In this animal 0.6 gram per kilo had apparently no effect whatsoever.

Experiment 7.—July 6. White rabbit, 1,610 grams.

11 a. m. Double nephrectomy; complete recovery.

2 p. m. Injected 0.6 gram magnesium sulphate per kilo in $\frac{M}{1}$ solution.

3.15. Some loss of muscular control, especially of hind legs; reflex much retarded.

5.15. Stretched out, completely relaxed; lid reflex sluggish but present; reacts promptly to pinch of tail.

8.15. Shows some recovery; able to sit up.

July 7, 9.30 a. m. No further recovery. General muscular weakness, especially of the anterior part, reacts sluggishly to stimulation, lid reflex retarded. Animal remained in the same state until it died July 8, 7 p. m. It lived fifty-three hours after the injection and fifty-six hours after the operation.

This animal was moderately but distinctly influenced by a dose of 0.6 gram per kilo. It recovered from a major part of the depressing influence within five hours after the injection, but retained some degree of depression until death which was apparently due solely to the nephrectomy.

These two experiments represent both extremes of the results. Out of seven experiments with 0.6 per kilo three have shown practically no signs of depression. In the other four animals anesthesia and paralysis were present in a variable degree, but were never complete and lasted only a few hours. In two the recovery was not complete.

In two experiments the injection of 0.6 gram per kilo was given about twenty-four hours after the nephrectomy. In one the de-

pression was hardly noticeable and in the other the depression was well marked, but of only short duration with complete recovery.

Apparently in nephrectomized rabbits a dose of 0.6 gram per kilo is bordering on the minimum toxic dose and the various uncontrollable minor factors which frequently influence to a slight extent the degree of absorption cause this dose to be sometimes without any effect and at other times to produce a well defined, though only temporary influence.

Cumulative Effect.—An interesting point is the question of the cumulative effect. In normal animals the effect of several subminimum doses administered at various times is not equal to the effect of the sum of these doses when given in single injection on account of the elimination which takes place during the intervals between the injections. It was different, however, with the behavior of magnesium salts in nephrectomized animals, as can be seen in the following experiment.

Experiment 8.—July 6, 1906. White rabbit, 1,740 grams.
12 noon. Double nephrectomy.
2.15 p. m. Injected magnesium sulphate 0.3 gram per kilo in $\frac{M}{1}$ solution.
3.30 and 4 p. m. Animal normal.
4.15. Injected again, 0.3 gram per kilo of the magnesium salt.
4.30. Animal lying on side, completely under anesthesia; lid reflex gone.
8.15. Seems somewhat improved; moves head.
8.20. Injected again 0.3 gram per kilo.
11 p. m. Animal profoundly under anesthesia.
July 7. 9 a. m. Somewhat improved; moves head and feet, but lid reflex still absent.
9.25. Injected again (fourth time) 0.3 gram per kilo.
12 noon. Profoundly under anesthesia; respiration very shallow.
Animal died at 2 p. m.

In this animal two subminimum doses of 0.3 gram per kilo given two hours apart had after the second dose at least as much effect as 0.6 gram per kilo given in one dose. A similar result was obtained when a dose of 0.2 gram per kilo was given hourly. About half an hour after the third dose the animal became fairly well anesthetized and recovered again after a few hours. In these cases, if the interval between the injections did not exceed two or three hours, the cumulative effect was perfect and in fact the sum of two or more doses seemed to be even more effective than a cor-

responding single dose. Possibly the cumulative effect might have been due to the fact that a single dose was given in only one place, while the several smaller doses were given in several different places, the latter circumstance favoring absorption.[10]

Discussion.—The following facts were brought out by the foregoing series of experiments.

A dose of one gram magnesium sulphate per kilo animal, when injected subcutaneously within two or three hours after double nephrectomy proved invariably to cause death a few hours after injection. In normal animals such a constant result could be obtained only with a dose of 2 grams per kilo.

A dose of 0.8 gram per kilo, injected within two or three hours after nephrectomy, was never fatal to the animal, but the animal became invariably more or less completely anesthetized and paralyzed. In normal animals such a constant effect could be attained only with a dose not less than 1.6 gram per kilo.

The susceptibility to the anesthetic and toxic effects of the magnesium salts was therefore in the nephrectomized animals increased with about fifty per cent. This increase of susceptibility is apparently due to the decrease in the facility of elimination of the salts by the urine. These experiments therefore tend to show that normally the kidneys carry off at least fifty per cent. of the injected salts. We should, however, lay little stress upon the exact figures. But we are, we believe, justified in stating that the experiments demonstrate conclusively *that the kidneys play an essential part in the elimination of the magnesium salts.*

In harmony with this conclusion is the observation which was made upon the cumulative action of the magnesium salt in the nephrectomised animals. The effect of the two or three injections of subminimum doses was at least equal to the sum of these quantities given in a single dose; a fact which can only be explained by the assumption that during the intervals none of the salt was eliminated. This observation would seem to contain the suggestion that at least during the first few hours after an injection no elimination takes place except through the kidneys. We shall, however, not dwell too much on this side of the question.

[10] Meltzer, *Jour. of Exper. Med.*, 1901, v, 643.

A further instructive fact is the observation that the profound anesthesia and paralysis produced by an injection of a dose of 0.8 gram per kilo lasted undiminished twelve to twenty hours and sometimes a good deal longer. Furthermore the recovery which finally took place was only moderate, the animal remaining until death in a pronounced state of paresis and stupor in sharp contrast with the nephrectomized control animal. In normal animals the anesthesia which was brought on by an efficient, but not fatal dose lasted at the utmost two hours and then the recovery was complete. This fact again can be best explained by the assumption that in the nephrectomized animal no fraction of the absorbed salts is eliminated for twelve or eighteen hours.

An interesting fact finally is the observation that a dose of one gram per kilo which is invariably fatal when given soon after nephrectomy is no longer fatal when injected eighteen to twenty-four hours after nephrectomy, although it still produces deep anesthesia; in other words eighteen to twenty-four hours after nephrectomy a dose of one gram per kilo acts like a dose of 0.8 per kilo injected soon after nephrectomy. This seems to indicate that some time after nephrectomy vicarious paths of elimination develop which thus assist in converting a fatal dose into only an anesthetic dose. This assumption would also explain the cause of the recovery from deep anesthesia twelve or eighteen hours after an injection of a dose of 0.8 gram per kilo; as at that time some of the salt becomes eliminated.

The degree of this vicarious elimination, however, is apparently very small; it never converted a fatal dose or an anesthetic dose into a harmless one, and the recovery from the anesthesia is only moderate, the animal generally remaining until death under a considerable influence of the salts.

The results which were obtained by us in the study of the effect of nephrectomy upon the toxicity of magnesium salts are in marked contrast to the results obtained by Meltzer and Salant[11] in their study of the effect of nephrectomy upon the toxicity of strychnin. Strychnin is generally assumed to be eliminated essentially through the kidney. Meltzer and Salant nevertheless found that the mini-

[11] Meltzer and Salant, *Journ. of Exper. Med.*, 1905, vi, 107.

mum toxic dose is for nephrectomized rabbits the same as for normal ones. For magnesium we found that for nephrectomized rabbits the dose is half of that which is toxic for the normal animals. Furthermore, the cumulative effect of strychnin is remarkably small. In intervals of two or three hours subminimum doses can be given, until they equal the sum of two or three times the toxic dose before any toxic symptoms will appear. For magnesium we found that the toxic effect will appear as soon as the sum of the subminimum doses becomes equal to the single minimum dose which is capable of producing an effect. Finally in nephrectomized rabbits no dose of strychnin was ever observed to produce continuous convulsions for any length of time; the animals either succumb soon or the convulsions gradually subside. With magnesium we observed that in nephrectomized rabbits the anesthesia may last uniformly for twenty-four hours and longer. Strychnin apparently finds soon after nephrectomy a satisfactory vicarious path for its elimination from the body, at least in rabbits. It should be mentioned that for guinea pigs Meltzer and Langmann[12] observed that within the first three hours after nephrectomy the toxic dose is indeed smaller than for the normal animal and that at that period in some animals a subminimum dose produced a continuous vibration which the authors termed a *subtetanic reaction*. They explained these observations by the assumption that in guinea pigs during the first few hours after nephrectomy vicarious paths for the elimination of strychnin are not yet developed.

Vicarious paths for elimination of magnesium even in rabbits do not develop until late after the nephrectomy and even then only in an unsatisfactory manner.

Conclusions.—Magnesium salts when introduced subcutaneously are eliminated to a great extent through the kidneys. In nephrectomized rabbits the susceptibility to the toxic effect of magnesium salts is increased by about fifty per cent.

The profound anesthesia which a toxic dose of magnesium produces in nephrectomized rabbits may be continuous for twenty-four hours and longer.

The cumulative effect of magnesium salts in nephrectomized

[12] Meltzer and Langmann, *Journ. of Med. Research*, 1903, ix, 19.

rabbits is very striking. The effect of several subminimum doses is equal to the effect produced by the sum of these doses given in a single injection.

A dose which when given soon after the nephrectomy is fatal, causes only a non-fatal anesthesia when given eighteen hours or later after the nephrectomy. Probably at that period vicarious paths develop sufficient for elimination of a fraction of the salts.

It is, probably for the last mentioned reason, that the profound anesthesia produced by a proper dose of the magnesium salts is partially recovered from about twelve to eighteen hours after nephrectomy.

THE INFLUENCE OF SALICYLIC ACID UPON URICOLYSIS.

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In a recent paper one[1] of us pointed out that subcutaneous injection of liver-extract (dog) might lead in the case of the dog to an increased power on the part of the liver to destroy uric acid in vitro. From these results it seems possible that the pharmacological action of some drugs might be connected with an increase in the rate of uricolysis. That the salicylates might act in this manner occurred to us as a possibility. The plausibility of this assumption relative to the action of salicylic acid seemed to be strengthened by the finding by one[2] of us that the liver, kidney, spleen and muscle taken from dogs treated with sodium salicylate showed a rate of uricocylis greater than that found in organs taken from normal dogs.

To test the accuracy of this view regarding the action of salicylates, dogs were injected subcutaneously (0.1 grm. sodium salicylate in one per cent. solution per kilo body weight) daily over periods of five and ten days, and the uricolytic power of the organs taken from the treated dogs was compared with that of organs taken from normal dogs under identical conditions. In all cases the dogs were bled to death, the organs removed as soon as possible and hashed finely, and a solution of sodium urate of known strength added to weighed portions of the macerated organs. Toluol was added to prevent bacterial action and the flasks were maintained at blood-temperature throughout the experiment. The uric acid remaining at the conclusion of the experiment was determined in duplicate by the method of Folin and Schaffer.[3] The average of the estimations is recorded.

[1] Stookey, L. B., *Jour. Med. Research*, 1906, xv, 321.
[2] Stookey, L. B., *Proc. Soc. Exper. Biol. and Med.*, 1907, iv, 59.
[3] Folin and Schaffer, *Zeit. für physiol. Chem.*, 1901, xxxii, 552.

Our results are contained in the following table.

No. of Dog.	Description of Dog.	Organ.	Grms. of Organ Taken.	Uric Acid Solution.		Length of Exposure in Hours.	Uric Acid Destroyed; in Grms.	Uric Acid Destroyed in Terms of 100 Grms. Organ in Grm.
				%	Vol. in c.c.			
I.	Normal.	Liver.	31	0.2	200	46	0.1210	0.3900
II.	Treated 10 days.	Liver.	20	0.2	150	44	0.0850	0.4250
III.	Normal.	Liver.	20	0.2	150	47	0.0743	0.3715
IV.	Treated 5 days.	Liver.	40	0.1	200	72	0.1685	0.4212
II.	Treated 10 days.	Liver.	20	0.2	150	67	0.1020	0.5100
III.	Normal.	Spleen.	11	0.1	150	47	0.0174	0.1581
II.	Treated 10 days.	Spleen.	11	0.1	150	44	0.0330	0.3000
II.	Treated 10 days.	Spleen.	9	0.1	150	67	0.0307	0.3416
I.	Normal.	Spleen.	13	0.1	150	67	0.0249	0.1921
III.	Normal.	Kidney.	27	0.1	150	46	0.0735	0.2722
II.	Treated 10 days.	Kidney.	19	0.2	150	44	0.1166	0.6138
III.	Normal.	Kidney.	27	0.1	150	70	0.0667	0.2472
IV.	Treated 5 days.	Kidney.	16	0.1	200	72	0.0750	0.4777
I.	Normal.	Kidney.	27	0.2	150	47	0.0660	0.2444
II.	Treated 10 days.	Kidney.	19	0.2	150	67	0.535	0.2815
II.	Normal.	Muscle.	20	0.1	150	47	0.0630	0.3000
II.	Treated 10 days.	Muscle.	20	0.1	150	44	0.0915	0.4575
III.	Normal.	Muscle.	65	0.1	200	46	0.0760	0.1169
III.	Normal.	Muscle.	58	0.1	200	70	0.0815	0.1404
II.	Treated 10 days.	Muscle.	20	0.1	150	67	0.0874	0.4370
I.	Normal.	Muscle.	58	0.1	200	144	0.0820	0.1413

An increased ability to destroy uric acid on the part of the kidney, muscle, spleen and liver taken from the dogs treated with sodium salicylate is noticeable.

Experiments regarding the influence of other agents upon uricolysis are in progress.

ON THE FUNCTION OF THE POSTERIOR SPINAL GANGLIA.

By S. P. KRAMER, M.D., Cincinnati.

In the course of an investigation on the nature of shock, certain phenomena concerning the influence of the spinal ganglia on the inhibition of shock were observed, and seem worthy of some special notice. In a previous publication[1] the author has described the effect upon the respiration and circulation produced by irritation of the central end of a divided sensory nerve, *i. e.*, the crural nerve, in dogs, and the variation in these effects with different degrees of anesthesia (ether).

During the experiments ether was administered through a tracheal tube. The blood pressure and cardiac contractions were recorded by means of a mercurial manometer, connected with the right carotid artery. The movements of the thorax were recorded by means of the exploring tambour of Paul Bert attached to a strap of adhesive plaster which encircled the lower portion of the chest. This tambour was connected through a rubber tube with a Marey tambour whose writing lever thus recorded the movements of expansion and contraction of the chest. The upstroke represented the inspiratory movement; the downstroke, the expiratory. The time was recorded by means of a metronome beating seconds placed in a circuit with an electrical signal, thus transmitting its rhythm to the signal, which marked the time on the paper of the kymograph.

The right crural nerve was divided and the central end stimulated electrically. For this purpose Du Bois Reymond's apparatus with platinum electrodes was used. An electric signal was placed in the primary circuit of the apparatus, so that the time during which the nerve was irritated was marked on the paper of the kymograph. Many experiments were made on the same animal in various stages of anesthesia. In only one dog was no anesthetic employed. The results here were constant and had been described before, so that I did not deem it justifiable to repeat this experiment.

[1] Kramer, *Annals of Surgery*, 1900, xxxii, 377.

Irritation of the central end of the divided crural nerve in an animal that had received no narcotic always produced the same effect. The respiration was increased in frequency about fifty per-cent. and the inspiratory effort was markedly increased. The cardiac rhythm was also increased approximately to the same extent. The blood pressure invariably rose. These effects gradually disappeared after the cessation of the stimulation, so that about one minute thereafter the respiratory rhythm and force were as before, and the same was true of the circulatory apparatus.

If the animals were deeply narcotized with ether, so that there was absolutely no corneal reflex, the result was very different. In most instances the irritation of the central end of the crural was without effect either upon the circulation or respiration.

At times there was a very slight effect on the respiration in that the inspiration was slightly inhibited. The rhythm remained unchanged and the circulation was not disturbed. There was however, a stage of ether anesthesia during which the effect was materially different. If the animal were anesthetized, but only partially, so that there was a condition of semi-anesthesia, in which the corneal reflex was present, but in which the animal lay quietly on the table, and if the cural nerve were irritated, the respiration, just as in unnarcotized animals, became greatly accelerated in rhythm and increased in amplitude. On the part of the circulatory apparatus, a very interesting change was observed. Instead of an increase in the pulse rate and a rise in pressure, as occurred in unnarcotized animals, we had a marked fall in blood pressure. This fall began a few seconds after the beginning of the irritation of the nerve, lasted for a varying number of seconds, and eventually returned to the level present before the irritation. The fall in blood pressure was greater the more intense the irritation, that is, the nearer the coils of the faradic apparatus were placed.

In every experiment there was a stage of general anesthesia in which irritation of the central end of the crural nerve produced this fall in blood pressure.

Experiments were also made upon animals narcotized with ether with a view of determining the influence of the posterior spinal ganglia on the transmission of these impulses. The posterior roots

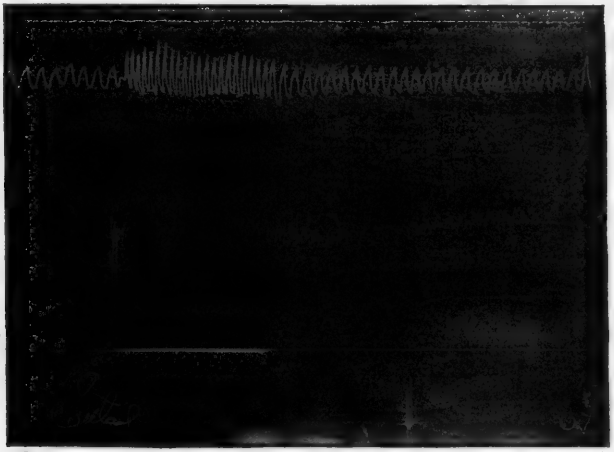
were exposed and one of the lower dorsal roots was divided peripherally to the ganglion; the electrodes were applied to the divided posterior root both centrally and peripherally to the ganglion. It was found that the spinal ganglion had an inhibitory effect. That is, a degree of irritation, which, in a given degree of anesthesia was without effect on the circulation or respiration when applied peripherally to the ganglion, would, when applied centrally, that is between the ganglion and the spinal cord, be followed by the "vasomotor shock," described above as following irritation of a sensory nerve during incomplete anesthesia. The spinal ganglion undoubtedly holds back or inhibits the effect of this irritation on its way to the cord and brain.

TRACING I. Electrode applied peripherally to the ganglion; anesthesia incomplete; corneal reflex present. Coils of faradic apparatus 10 c.m. apart.

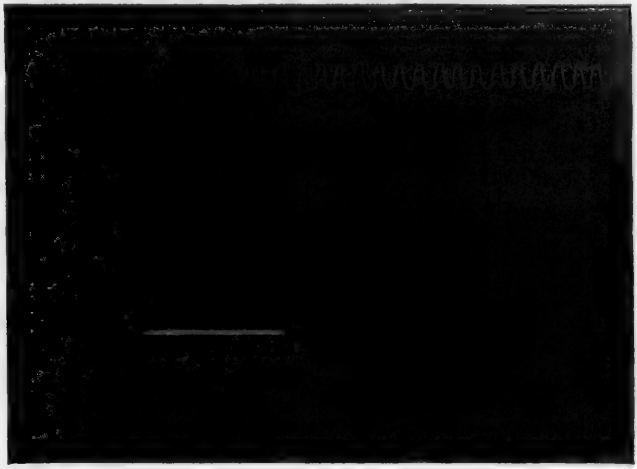
Tracings I and II are records of two experiments on the same dog made about a minute apart. The animal was anesthetized with ether; the corneal reflexes were present. The coils of the faradic apparatus were ten centimeters apart.

Tracing I is a record of an experiment in which the electrodes were applied to the spinal root peripherally to the ganglion. There

is a slight increase in amplitude of the respiratory movements and practically no effect upon the circulation.

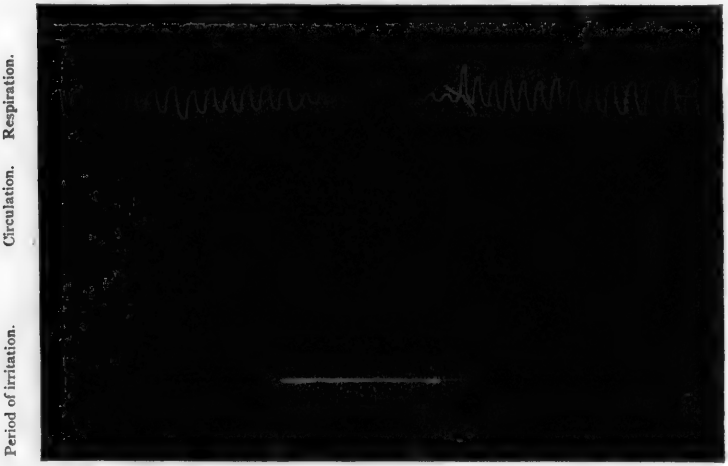


TRACING 2. Electrode applied centrally to the ganglion; anesthesia incomplete; corneal reflex present. Coils of the faradic apparatus 10 c.m. apart.



TRACING 3. Electrode applied peripherally to the spinal ganglion; complete anesthesia; no corneal reflex. Coils of faradic apparatus 5 c.m. apart.

Tracing II is a record of an experiment in which electrodes were applied centrally to the ganglion. We have here the effect noted before, namely a marked acceleration of rhythm and increase in amplitude of the respiratory movements, and a decided fall in blood pressure. These experiments show that an amount of irritation which, when applied to the posterior roots peripherally to the ganglion had produced practically no effect, when applied centrally to the ganglion was followed by marked "vaso-motor shock." A similar result was obtained in deeply narcotized animals in which the corneal reflex was absent, and where stronger stimuli were applied.



TRACING 4. Conditions are the same as with Tracing 3. Electrode applied centrally to the spinal ganglion.

Tracings III and IV are records of two experiments on the same dog in which the electrodes have been applied peripherally and centrally to the ganglion, the coils being five centimeters apart. The tracings show the same thing as before, that is, little or no effect when the electrodes are applied peripherally and marked "vaso-motor shock" when applied centrally to the ganglion.

It is believed that the registration of these phenomena will provide a valuable method for studying the functions of the spinal ganglia under various conditions. The author intends at some future time to report the results of his researches along this line.

THE PATHOGENESIS OF GALL STONES.

By S. P. KRAMER, M.D., Cincinnati.

The ultimate cause for the formation of gall stones has, notwithstanding the large amount of investigation on the subject, remained a subject for speculation.

The earliest view was a purely mechanical one; that the formation of the stones was the result of concentration due to stagnation. On account of the fact that bile may be concentrated to a thick mass without the precipitation of the products found in gall stones, this view had to be abandoned.

There then followed the so-called chemical theory, the chief exponent of which was Thudichum.[1] Cholestearin, bilirubin and the calcium salts are soluble in alkaline solution, especially in solutions of sodium glycocholate, and concentration does not bring about precipitation. If, however, according to Thudichum, as the result of decomposition due to prolonged standing or as the result of abnormal secretion of mucus, the bile becomes acid, the cholestearin and bilirubin calcium are precipitated as the result of the decomposition of the solvent, sodium glycocholate, into glycocoll, cholalic acid and a sodium salt. In reading the work of Thudichum, it is well to remember that the work was done a half century ago, and of course, without bacterial investigation.

Another chemical explanation was offered by Dochmann[2] in 1891. He analysed both liver bile and bladder bile, the latter obtained after ligation of the cystic duct, and found a great increase in calcium and diminution in sodium in bladder bile as compared with liver bile. According to his view the increase in calcium diminishes the solubility of bilirubin and leads to a precipitation of bilirubin calcium and cholestearin. Prolonged intervals between meals and stasis cause a stagnation and precipitation of bilirubin calcium and cholestearin.

[1] Thudichum, *Quarterly Journal of the Chemical Society*, 1862, xiv, 114.

[2] Dochmann, *Wien. med. Presse*, 1891, xxxii, 1198.

Opposed to these chemical theories are the morphological and bacteriological theories. Dujardin Beaumetz[3] ascribes the formation of gall stones to a desquamative cholangitis and biliary stasis, thus reviving the "Stein bildende Katarrh" of Meckel.[4]

Naunyn[5] denies the chemical theory of Thudichum. Even when greatly concentrated, bile contains sufficient solvent to keep the cholestearin and bilirubin calcium in solution. The decomposition of sodium glycocholate to sodium cholate does not explain the precipitation, since this product is also a solvent for cholestearin and bilirubin calcium. He also ascribes the formation of gall stones to a desquamative cholangitis.

This inflammatory process leads to a desquamation of epithelial cells which degenerate and form cholestearin and calcium salts. The bilirubin unites with the calcium. The precipitation of bilirubin calcium is aided by the albuminous character of the products of cellular degeneration, alluding here to the general tendency of albumen to precipitate calcium.

In this way are formed small particles which form the nuclei of gall stones. The cholestearin is deposited later, partly on the outside and partly infiltrating the mass through the so-called infiltration canals which are found in gall stones. The mere excess of calcium will not cause a precipitation of bilirubin in calcium and cholestearin. Even the presence of foreign bodies as centers of crystallization will not bring this about.

There is lacking the explanation of some biological process which brings this about, and this, according to Naunyn, is furnished by the degeneration of epithelial cells the result of an infective cholangitis.

We have still, however, no explanation as to the process by which cholestearin and calcium salts, which are normally soluble in bile, are precipitated in cholelithiasis. To say that it is the result of degenerative changes in epithelium, produces more mystery, but no explanation.

If now we leave the realm of speculation and search for some exact knowledge, a very curious chain of thought will arise. We

[3] Dujardin Beaumetz, *Bul. Therap.*, 1891, cxxi, 291.
[4] Meckel von Helmsbach, "Mikro-geologie," 1856, Berlin.
[5] Naunyn, "Klinik der Cholelithiasis," 1892.

have, heretofore, exact knowledge of the formation of stones in one instance and in one only. I refer to the formation of phosphatic urinary calculi. We know that this is due to chemical decomposition, the direct result of bacterial growth. If now we apply this knowledge to experiments, having for their object the clearing up of the subject of gall stones, it may be that some success will follow. It has been abundantly proven, that bacteria are at all times present in gall stones. The literature on the subject is so well known that it is not necessary to burden this article with it.

The colon bacillus is the organism most frequently found in gall stones. The one next in frequency is the typhoid bacillus. It is very probable that it will be found that infection by one of these two micro-organisms is responsible for most cases of cholelithiasis.

Now while this has been commonly accepted, it seems strange that there should be no investigation published, showing the effect of these organisms on bile when grown in it.

It would appear *a priori* reasonable, that possibly the growth of these organisms in bile, or a solution of bile, would effect the precipitation of some of the biliary constituents. Accordingly, for the past year I have been carrying out such an investigation with most gratifying results.

Culture tubes were prepared containing a mixture of one half human bile obtained at autopsy and one half ordinary alkaline pepton bouillon. This mixture or solution was repeatedly sterilized and filtered, until a perfectly clear medium was obtained. Such tubes were inoculated with the colon bacillus, the typhoid bacillus and Staphylococcus pyogenes aureus. All of these micro-organisms grow readily in this medium. The staphylococcus forms a copious growth which sinks to the bottom of the tube, the medium showing no apparent change.

Not so, however, with those inoculated with the colon or typhoid bacillus. Here in a few days the medium became cloudy and a precipitate is seen at the bottom of the tube. This precipitate increases greatly until in about four weeks a very well marked, closely packed, semi-solid mass is seen at the bottom of the tube. If allowed to incubate longer, say for six months, care being taken to prevent evaporation by rubber caps, this precipitated mass becomes

firmly packed and the super-natant fluid may be poured off and we have a kind of very soft "gall stone" as it were, taking the form of the bottom of the tube. If we examine the precipitate, we have no difficulty in recognizing all the constituents of gall-stones as well as masses of bacilli: Amorphous calcium phosphate, magnesium phosphate, calcium carbonate, biliary coloring matter and a few crystals of cholestearin. Crystals of ammonio-magnesium phosphate are formed very late, usually after many weeks of growth.

In order that the precipitation of cholestearin might be made more manifest, and since, normally, bile contains comparatively

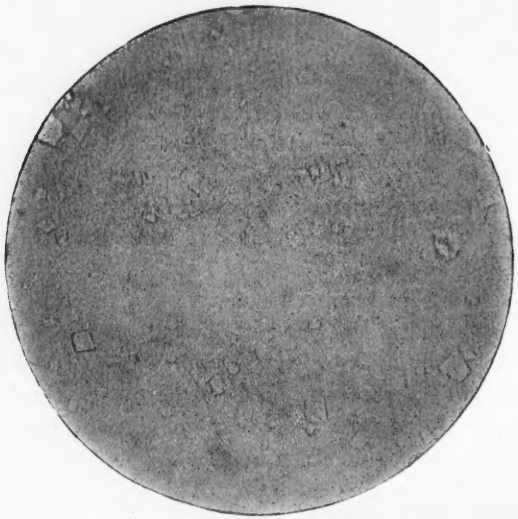


FIG. 1. Photo-micrograph of precipitate in culture of *Bacillus coli,* in medium containing 50 per cent. bile and 1 per cent. cholesterin. Culture sixty days old.

little of it, culture tubes were prepared in which cholestearin was added to the solution of bile and bouillon. This readily dissolves therein and the medium was carefully filtered and examined microscopically before inoculated, so as to be sure that all the cholestearin present was in solution. Such tubes when inoculated with

colon or typhoid bacillus, give a precipitate very rich in cholestearin crystals, as may be seen from Fig. 1, a photomicrograph of such a precipitate.

Another thing which was remarked, was the markedly preservative action of bile upon colon and typhoid bacilli. I have at present tubes in which the precipitate was separated and allowed to dry out completely, and yet the bacilli present are still viable and readily resume growth when inoculated upon fresh media. This corresponds with the findings that viable typhoid bacilli have been found in the interior of gall stones years after the individual had passed through the attack of typhoid fever.

Thus Droba[6] reports a case in which he obtained the typhoid bacillus from gall stones removed seventeen years after the patient had recovered from typhoid fever.

It appears to me that these experiments indicate very clearly then that gall stone formation, just as phosphatic urinary stone formation, is due to a chemical decomposition of the bile, the direct result of the growth of micro-organism therein. Bacillus coli communis, and Bacillus typhosus are the micro-organisms usually concerned. Just what the exact chemical nature of the decomposition is, remains to be shown. It is rather significant, however, that the two micro-organisms which cause this precipitation in vitro, produce an acid reaction in the media. Whereas, the staphlococcus, which requires and retains in akaline reaction, does not cause the precipitation.

It may be then that the decomposition theory of Thudichum will prove to be true in a modified form. His work on the decomposition of bile was done, of course, before the days of pure culture, and was without bacterial control. A repetition of his analyses of bile decomposed by pure culture of these organisms, may give us the exact chemical nature of the process.

[6] Droba, *Wien. klin. Woch.*, 1899, xii, 1141.